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## Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. Collaborative work, in particular with regard to systematic reviews, is acknowledged throughout the thesis. All systematic reviews were completed as a team with screening and data extraction completed by myself and another reviewer independently. This thesis was entirely written as my own work in conjunction with my supervisor, Professor Molloy.

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A handwritten signature in black ink that reads "Tim Kenley". The signature is written in a cursive style with a long horizontal stroke at the beginning and a large loop at the end.

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## Abbreviations

AAP: American Academy of Pediatrics

ACOG: American Congress of Obstetricians and Gynecologists

aEEG: amplitude-integrated EEG

ANOVA: analysis of variance

ASC: apoptosis-associated speck-like protein

BBB: blood brain barrier

BE: base excess

BG/T: basal ganglia / thalamus

BMAL1: Brain and Muscle Arnt-Like 1

CI: confidence interval

CLOCK: circadian locomotor output cycles kaput

CNS: central nervous system

CP: cerebral palsy

CR: circadian rhythm

CRG: circadian rhythm genes

CRP: c-reactive protein

CRY: cryptochrome

CSF: cerebrospinal fluid

cytC: cytochrome C

D/PAMP: damage/pathogen associated molecular patterns

DEF: data extraction form



DWI: diffusion weighted imaging

EEG: electroencephalogram

ELISA: enzyme linked immunosorbent assay

Epo: erythropoietin

FSC: forward scatter

GA: gestational age

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte and macrophage colony-stimulating factor

GMFCS: gross motor function classification scale

HI: hypoxia ischaemia

HIE: Hypoxic ischaemic encephalopathy

HIF: hypoxia inducible factor

IL: interleukin

IFN: interferon

IM: intramuscular

IQR: interquartile range

IV: intravenous

LBW: low birth weight

LDH: lactate dehydrogenase

LPS: lipopolysaccharides

miRNA: microRNA

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

NDD: neurodevelopmental disability

NE: neonatal encephalopathy

NF-kB: nuclear Factor kappa B

NICHHD: National Institute of Child Health and Human Development

NICU: neonatal intensive care unit

NLRP3: NOD-, LRR-, and pyrin domain-containing protein 3

NOX: nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase

OR: odds ratio

PA: perinatal asphyxia

Pam3Csk4: Pam3Cys-Ser-(Lys)<sub>4</sub>. Trihydrochloride

PBA: phosphate buffered alanine

PBS: phosphate-buffered solution

PLIC: posterior limb of the internal capsule

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PRR: pathogen recognition receptor

PSE: perinatal sentinel event

qPCR: quantitative polymerase chain reaction

RCT: randomised controlled trial

ROB: risk of bias

ROS: reactive oxygen species

SCN: suprachiasmatic nucleus

SQ: sleep quality

SSC: side scatter

SWC: sleep wake cycling

TGF: transforming growth factor

TH: therapeutic hypothermia

TLR: toll-like receptor

TNF: tumour necrosis factor

VEGF: vascular endothelial growth factor

VON: Vermont-Oxford Network

WCC: white cell count

WM: white matter

## Abstract

**Background:** Neonatal Encephalopathy (NE) is a clinically defined syndrome of disturbed neurological function in the earliest days of life in term and near-term newborns. NE remains a major cause of mortality and morbidity in the neonatal population, but remains poorly defined. An increasing number of interventions are available for infants at risk of adverse neurodevelopmental outcome, but prognostication remains a significant challenge with diagnostic uncertainty with MRI and biochemical biomarkers. Dysregulated immune function is associated with worse outcome in NE. Circadian rhythms (CR) have an important role in the regulation of the immune system. Studies have shown that infants with NE and dysregulated CRs have worse outcomes. We investigated different diagnostic criteria of NE, the prognostic value of MRI and different biomarkers in NE, the evidence for melatonin treatment for those with NE, and the influence of melatonin on systemic inflammation in NE.

**Methods:** Systematic reviews were conducted according to PRISMA or Cochrane guidelines. Patients with NE and age-matched controls were recruited during the first week of life and blood samples obtained. Samples were examined ex-vivo to assess the response in serum cytokine production, inflammasome gene and circadian gene expression, neutrophil and monocyte cell marker expression, and microRNA gene expression to melatonin treatment and LPS stimulation. Sleep quality and sleep wake cycling was analysed and correlated with measures of systemic inflammation.

**Results:** NE remains poorly defined and inconsistent diagnostic criteria are applied. A significant but unclear distinction between NE and hypoxic ischaemic encephalopathy (HIE) persists despite many attempts over recent years. MRI provides superior prognostic accuracy compared to other techniques, and MR spectroscopy provides the best prognostic value overall. Melatonin appears to provide neuroprotection in NE, however the evidence to date is very uncertain. Melatonin is a potent immune regulating agent in infants with NE, although it induces a complex immune response which requires further evaluation.

**Conclusion:** Lack of a clear definition of NE, consistent use of terminology, and variation in diagnostic criteria limits research into the condition and requires consensus approach as efforts to date have not resulted in greater uniformity in understanding or terminology use. A wide variety of biomarkers and other prognostic markers have been investigated in recent years. MR spectroscopy provides the most accurate prognostic value but requires further evaluation for best implementation. Phase III studies of melatonin treatment are urgently required as it appears to provide neuroprotection in NE but evidence is very limited to date. Melatonin is a potent immune-modulating agent in NE, however further investigations into these effects are required.

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## Chapter 1: Introduction

### Neonatal encephalopathy

Neonatal encephalopathy is generally understood to be a clinically defined syndrome of disturbed neurological function in the first few days of life [1]. It has a multifactorial aetiology but a final common pathway of hypoxia-ischaemia that leads to a cascade of events which result in cell death [2]. NE remains one of the most common causes of global neonatal mortality [3]. Therapeutic hypothermia is the only neuroprotective intervention for NE and it reduces mortality and long-term neurodisability in survivors [4]. However evidence suggests that TH is not safe nor feasible in low-middle income countries where NE is most prevalent [5]. There is no robust evidence for the use of TH and it is not routinely used for those with mild NE who are also at risk of adverse outcome [6].

### Epidemiology of neonatal encephalopathy

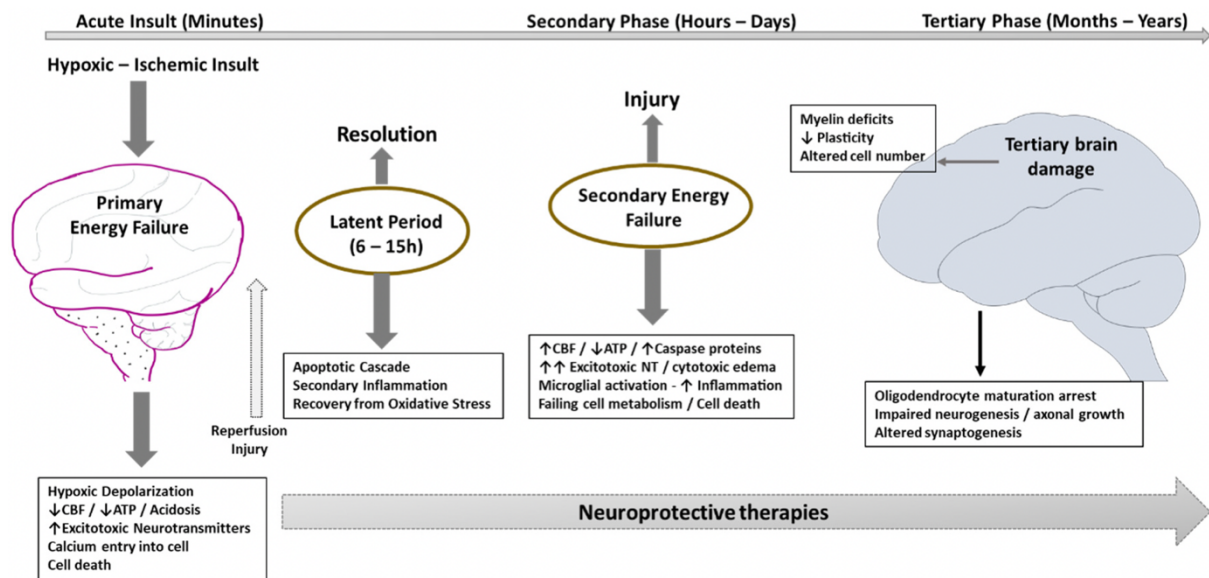
The World Health Organisation identifies NE as one of the ten leading causes of lost years of life [7]. However, the lack of an agreed terminology or consensus definition of NE causes significant difficulties estimating the incidence and prevalence of the condition [8-10]. A variety of terminology is used for the condition including NE, hypoxic ischaemic encephalopathy (HIE), birth or perinatal asphyxia, or birth injury. NE is a syndrome which is diagnosed clinically so there is no diagnostic test or criteria used to define the condition. Therefore there are wide variations in the estimated incidence and prevalence of the condition. A large systematic review estimated the global incidence of NE to effect 1.15

million infants annually (uncertainty range: 0.89–1.60 million) with an incidence of 8.5 cases per 1,000 live births and 96% of cases occurring in low to middle income countries [11]. Other estimates of the incidence of NE are 3.0 per 1000 live births (95%CI 2.7 to 3.3) and HIE is estimated to be 1.5 per 1000 live births (95%CI 1.3 to 1.7). While some argue HIE is a cause-specific subset of those with NE, there is overlap in use of the terms and they are frequently used interchangeably [8]. More recent estimates suggest that moderate-severe NE affects 0.5-3.0 per 1000 live births in high-income countries. Estimates in low-middle income countries were much higher, however their definitions of NE were much different to those in high-income countries making comparisons more difficult [12]. Several studies have reported reducing global incidence of NE in recent years [11] and in particular in high income countries [13-15], while using the same definition. The national incidence of NE requiring TH in Ireland from 2016-2019 was 1.1 per 1000 live births [16].

### Pathogenesis of Neonatal Encephalopathy

As mentioned, NE is a clinical diagnosis and has a multifactorial aetiology [2]. Although in many cases the causative event in NE cannot be identified, there is a final common pathway triggered by reduced cerebral perfusion and hypoxia [17]. This leads to a cascade of events that result in neuronal cell death in the following hours and days, and prevention of repair and regeneration over the following months and years [18]. The events that result in cell death are characterised by excitotoxicity, dysregulated systemic inflammation, oxidative stress, mitochondrial energy production failure, and apoptosis [19]. From experimental animal models, 3 stages of injury in NE have been identified (**Figure 1**).

Although there is significant overlap between the mechanisms of cell death, different mechanisms predominate during individual stages [20]. Recognition of the different phases of injury, and the different underlying mechanisms provides opportunities to provide effective interventions at different time points of the injury.

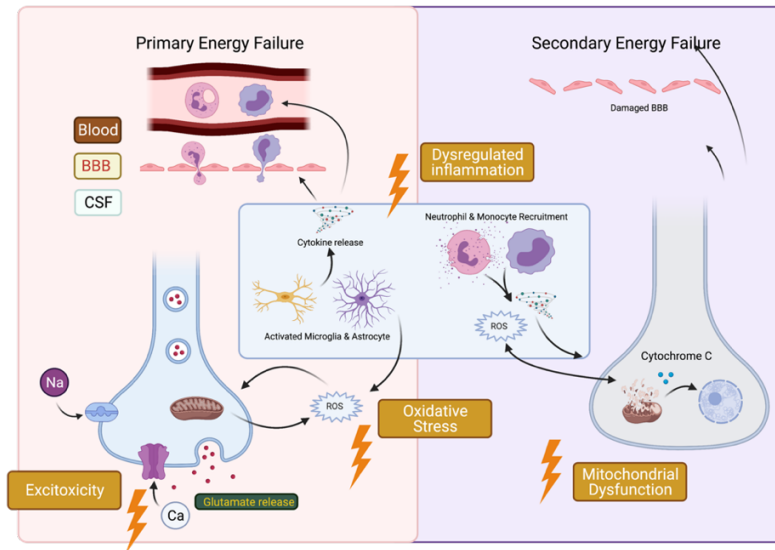


**Figure 1 Phases of injury in neonatal encephalopathy, from Nair et al, Children, 2018 [20].** Schematic illustration of pathophysiology of HIE in relation to hypoxic ischemic (HI) insult resulting in primary (acute phase) and secondary energy failure (secondary phase) in the brain. Brain resulting in primary (acute phase) and secondary energy failure (secondary phase) in the brain. Brain damage (tertiary phase) continues to occur months to years after the injury resulting in decreased plasticity and reduced number of neurons. Latent period following resuscitation is ideal for interventions to decrease the impact of secondary energy failure. However, strategies are developed to attenuate tertiary brain damage which will expand the therapeutic window, substantially increasing the beneficial effects of neuroprotection in these infants and hence its impact on long-term outcomes. CBF—cerebral blood flow; ATP—Adenosine tri phosphate; NT—neurotransmitters.

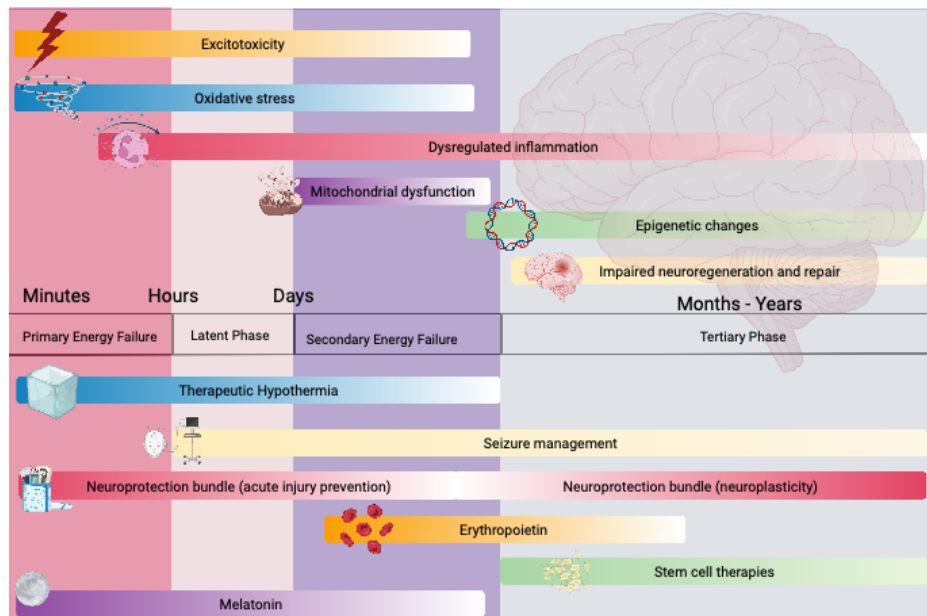
## Stages of cell death

The first 2 phases of injury occur during the first 2 weeks following hypoxic ischaemic (HI) injury (Figure 2). Primary energy failure phase, or primary neuronal death, can occur within minutes or up to 6 hours after the initial HI insult. During this stage reduced cerebral perfusion causes depletion of oxygen, glucose, and adenosine triphosphate (ATP) energy reserves, and excess lactate production leading to systemic acidosis. These changes result in failure of the neuronal cell membrane. This causes excessive neuronal release of excitotoxic neurotransmitters with massive calcium ( $\text{Ca}^{2+}$ ) influx into the cell and necrotic cell death, activation of microglia within the central nervous system (CNS), and breakdown of the blood brain barrier (BBB). There are no therapies currently available to target mechanisms of injury during this phase and early recognition of injury and resuscitation remain the most important interventions [21]. A latent phase follows, with the return of normal cerebral perfusion. The duration of this phase is inversely proportional to the severity of injury [22]. Reperfusion injury, or secondary energy failure phase, occurs 6-15 hours following the HI insult and can last for hours to days [19]. During this phase the majority of cell death occurs as a result of dysregulated inflammation, oxidative stress, mitochondrial dysfunction, excitotoxicity, and the initiation of apoptotic cell death pathways [20]. It is this phase of injury that therapeutic hypothermia aims to disrupt and prevent and a number of anti-inflammatory and anti-oxidant therapies are under investigation. Tertiary phase has been recognised more recently and is characterised by persistent dysregulated inflammation and epigenetic changes [18]. To date most evidence of injury in this phase is from experimental models, however more direct evidence

of dysregulated inflammation is emerging [23]. Recognition of this phase of injury may provide a further therapeutic window for neuroprotective interventions but none are currently used in routine practice.



**Figure 2 Schematic representation of the mechanisms of injury during the early phases in neonatal encephalopathy. Created with BioRender.com.**

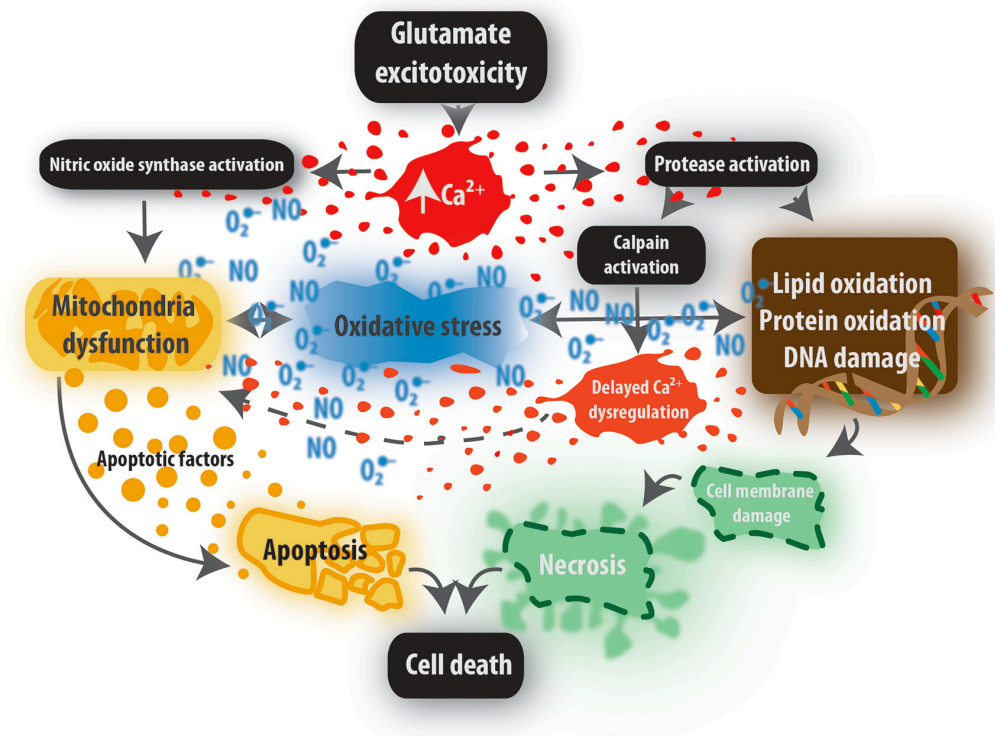


**Figure 3 Phases of injury in neonatal encephalopathy and proposed treatment. Created with BioRender.com.**

## **Role of excitotoxicity**

The depletion of ATP resources following HI insult results in dysfunction of the neuronal active transport  $\text{Na}^+/\text{K}^+$  transmembrane pump [24]. This dysfunction results in intracellular  $\text{Na}^+$  influx, extracellular accumulation of  $\text{K}^+$ , membrane depolarisation, and opening of the voltage-gated  $\text{Ca}^{2+}$  channels [25]. Massive intracellular  $\text{Ca}^{2+}$  accumulation triggers the uncontrolled release of the excitatory neurotransmitter glutamate. Excess glutamate release activates the calcium-permeable ionotropic NMDA receptor (NMDAR) and causes overexcitation of neurons post-synaptically [26]. Glutamate excitotoxicity results in further intracellular  $\text{Ca}^{2+}$  uptake and impaired glutamate uptake by astrocytes, which results in a vicious cycle causing massive intracellular  $\text{Ca}^{2+}$  accumulation. The excessive intracellular accumulation of  $\text{Ca}^{2+}$  leads to the activation of  $\text{Ca}^{2+}$ -dependent enzymes, including proteases and nitric oxide synthase (NOS). This causes mitochondrial dysfunction, oxidative stress, and oxidation of essential macromolecules, and increased production of pro-apoptotic proteins which all contributing to cell death by apoptosis or necrosis [25]. Following hypoxia-ischaemia spreading depolarizations (SDs) occur abundantly and result in an electrochemical membrane failure and neuronal swelling [27]. SDs are characterised by a 'rapid and strong neuronal and glial depolarization and near-complete breakdown of the ionic gradients between the extracellular and the intracellular compartment due to a net influx of sodium and calcium into the cells, releasing of potassium and cellular edema' [28]. SDs result in cell death in viable tissue in the ischaemic penumbra and loss of viable neuronal tissue by spreading depression [29].





*Figure 4 Excitotoxic events caused by aberrant  $Ca^{2+}$  levels, from Ischemia-Triggered Glutamate Excitotoxicity From the Perspective of Glial Cells, Belov et al, Frontiers in Cellular Neuroscience, 2020 [25]. High concentrations of intracellular  $Ca^{2+}$  lead to the activation of  $Ca^{2+}$ -dependent enzymes, such as proteases and nitric oxide synthase (NOS). This results in the dysfunction of mitochondria, oxidative stress, and oxidation of essential macromolecules, all contributing to apoptosis or necrosis.*

### Dysregulated inflammation

CNS inflammation is critical process of normal development and response to injuries such as NE [30]. Activation of inflammatory processes protects from invading microbes and contributes to regeneration of viable tissue but at the cost of ‘bystander’ brain injury [27]. However dysregulated inflammation in the perinatal period increases the risk of mortality and long-term neurodevelopmental disability. The newborn brain is uniquely vulnerable to neuroinflammation due to higher oxygen and glucose consumption, low concentration of

antioxidant enzymes, low myelination, and higher concentrations of free iron [30]. The central nervous system is an immune privileged site which requires protection from damage from the peripheral immune cells as it has limited capacity for regeneration [31]. The CNS is protected from the peripheral immune cells by the blood brain barrier (BBB), but protection from the BBB is undermined during periods of neuroinflammation [32]. As discussed above, regardless of the cause of NE there is final common pathway that leads to hypoxia and ischaemia (HI). HI leads to activation of the innate immune system both within the CNS, and in the peripheral immune system.

Microglia, resident immune cells in the CNS, are the first responders following HI injury [33]. Following ischaemic brain injury, microglia initiate ROS generation, phagocytosis and the production of inflammatory mediators including IL-1 $\beta$ , TNF $\alpha$ , IL-6 and MMPs. HI insult also causes damage to endothelial cells of the BBB are due to excessive glutamate from energy failure, and free radicals [34]. Microglial activation and oxidative stress further increase the permeability of the BBB [35]. Increased permeability of the BBB leads to infiltration of peripheral immune cells. Microglia also interact with infiltrating leukocytes from the periphery. Several cells of the peripheral innate immune system have been implicated in brain injury in NE, in particular neutrophils and monocytes.

Neutrophils are the first cells recruited to site of injured tissues by the release of DAMPS and chemokines [36]. Neutrophil production in the bone marrow is stimulated by granulocyte colony-stimulating factor (G-CSF), which is increased in response to tissue injury

[37]. While neutrophils and monocytes have important roles in host defence against invading pathogens, they can also damage surrounding or inflamed tissue. Neutrophils cause cell death by degranulation with the extracellular release of cytotoxic proteins, proteases, and reactive oxygen species (ROS), and the formation of neutrophil extracellular traps (NETs) [38]. Monocytes are central mediators of cell death and cause cell death by phagocytosis and are potent secretors of inflammatory cytokines [39]. Neutrophil degranulation is enhanced in hypoxic conditions, and these changes occur rapidly [40]. While neutrophils are early responders to tissue injury, they are usually sequestered within the blood vessels for a prolonged period and injure endothelial cells through NET-related mechanisms [41]. Higher neutrophil count in the first 4 days of life is associated with adverse outcome in NE [42]. NE is associated with dysregulated neutrophil and monocyte functional phenotype [43], persistent neutrophil and monocyte activation [44], and delayed neutrophil apoptosis [45]. Induced neutropenia has demonstrated neuroprotection in animal models of neonatal asphyxia [46].

### **Oxidative stress**

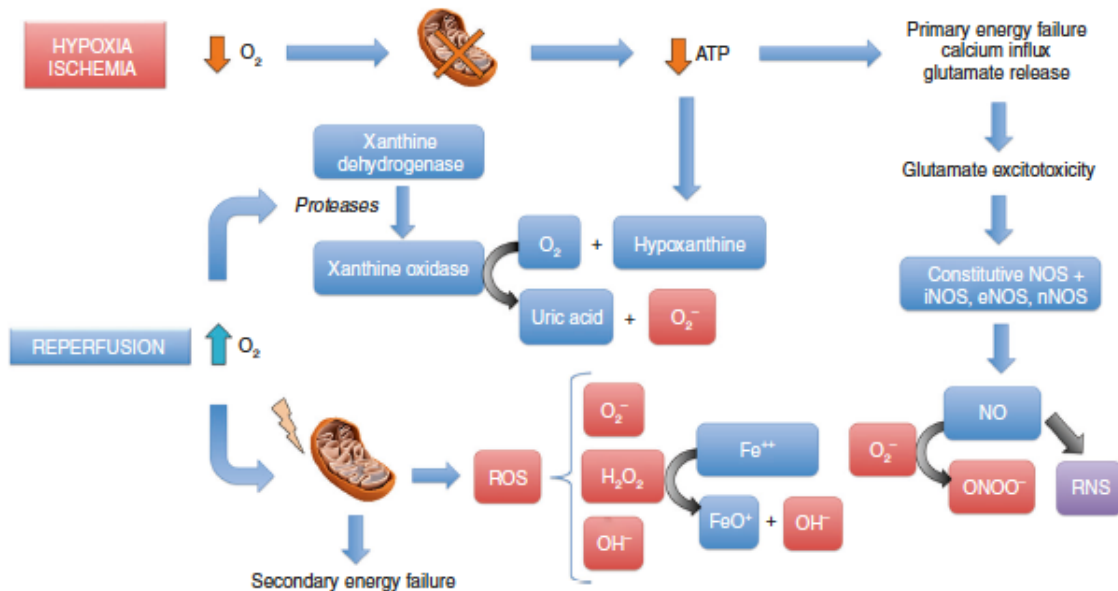
The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is an important part of host antimicrobial defence. However, when produced in excessive amounts ROS and RNS can overwhelm antioxidant defence mechanisms resulting in oxidative stress (OS), which can cause significant tissue damage to biological macromolecules including proteins, lipids and nucleic acids. Relative OS is generally high in the immediate postnatal period as the newborn adjusts to the higher oxygen environment,

however the addition of acute asphyxia and free iron increases the susceptibility to injury by OS [47]. Due to immature antioxidant defences and increased cell sensitivity to oxygen free radicals OS can be particularly damaging during the neonatal period. A wide variety of neonatal pathologies have been linked to OS, including NE [47]. The newborn brain is particularly vulnerable to OS due to high metabolic demands, low concentrations of anti-oxidant enzymes, a high proportion of fatty acids, and a high concentration of free iron [48].

Excessive ROS are produced at several different stages of injury in NE. During HI, neutrophils are the primary source of ROS, although other cell types including monocytes contribute to their production, albeit to a much lesser degree [44]. The key ROS produced during HI & reperfusion are NO, superoxide radical anion  $O_2^-$ , which react to form the powerful oxidant peroxynitrite ( $ONOO^-$ ), hydrogen peroxide and hydroxyl free radical OH. In the PEF stage HI leads to energy failure from adenosine triphosphate (ATP) depletion and systemic acidosis due to lactate accumulation [20]. ATP depletion causes failure of the ATP-dependent pumps which causes intracellular accumulation of  $Ca^{2+}$  and the release of neurotransmitters such as glutamate, stimulating AMPA and NMDA receptors [48]. These changes, along with accumulated lactate and extracellular glutamate, result in excitotoxicity of cells and the generation of ROS and RNS [17]. Activation of NMDA glutamate receptors stimulates synthesis of NO via the NOS system. These changes cause a loss of integrity of the cell membrane with the intracellular accumulation of  $Na^+$  and  $Ca^{2+}$ , and result in further ROS production, cytotoxic oedema and cell death by necrosis [19]. In ischaemic injury, NADPH oxidase (NOX) enzymes are unique as they are solely responsible for ROS production

[48]. Following the latent phase, reperfusion and an extension of injuries from the PEF result in the majority of cell death during the SEF phase. Injuries from PEF stage including OS cause mitochondrial changes during the SEF. While mitochondria themselves are susceptible to damage from OS, mitochondrial dysfunction is also the major contributor to further production of ROS. ROS and RNS cause neuronal cell death through several mechanisms. ROS causes intracellular accumulation of  $Ca^{2+}$  as described above, leading to death by necrosis. ROS also leads to the reduced integrity of the BBB by activation of xanthine oxidase, increased neuronal expression of pro-apoptotic genes [49], increased generation of the toxic free radical peroxynitrite which interferes with the cell membrane further increasing the expression of pro-apoptotic genes including cytochrome C [50], and increased lipid peroxidation and DNA damage [51]. OS also leads to impaired glucose metabolism and further acidosis as a result of lactate accumulation [52]. As a result of recognition of the contribution of OS to injury in NE, OS has become a major target for further therapies in NE [21]. OS and dysregulated inflammation are known to interact to create a 'window of susceptibility' in NE [48]. For example, selective inhibition of neuronal NO synthase provides neuroprotection in a model of cerebral palsy [53]. The close link between ROS and inflammation was first understood when the oxidative burst of PMNs was described almost 50 years ago [54]. Activated inflammatory cells release a large amount of oxygen radicals and proteases, resulting in direct tissue injury, while also leading to production of pro-inflammatory cytokines. Inflammatory cytokines can ultimately be a source of excessive ROS production with subsequent damage to innate immune cells and further endothelial dysfunction [47]. Therefore, the consequence of excessive inflammation appears to be a

'vicious cycle' whereby oxidative stress induces cytokine release, which in turn further increase ROS production [47].



**Figure 5 Mechanisms of free radical production following hypoxia-ischaemia and reperfusion, from *Free radicals and neonatal encephalopathy: mechanisms of injury, biomarkers, and antioxidant treatment perspectives*, Martini et al, *Pediatric Research*, 2019 [21].**

### Role of mitochondrial dysfunction

Mitochondrial alteration plays a crucial role in the pathogenesis of NE and interacts with all other mechanisms of injury. Following HI there is increased permeability of the inner mitochondrial membrane causing disruption of oxidative phosphorylation in the electron transport chain (ETC), ATP production deficiency and consequently increased anaerobic metabolism [21]. The consequences include further glucose depletion and energy

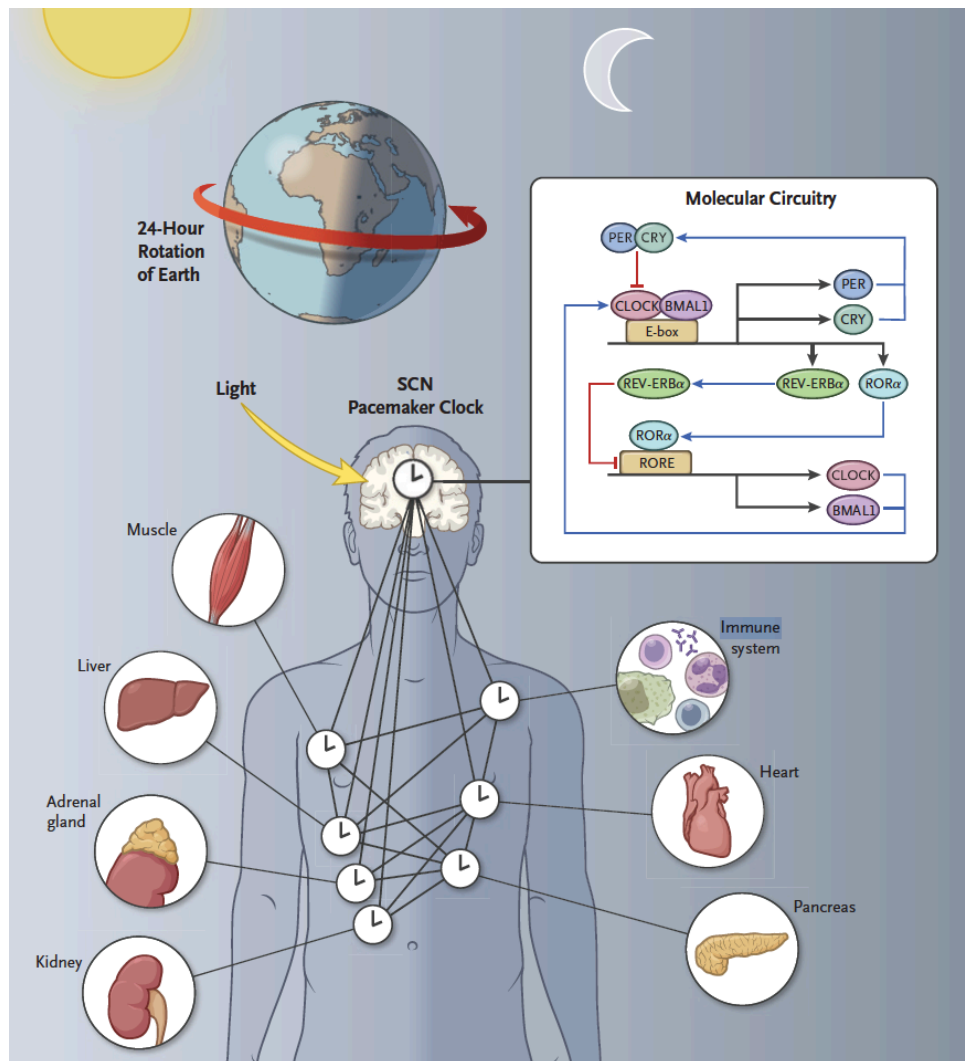
failure, worsening lactate production and further systemic acidosis, release of ROS, increased production of apoptotic proteins. Energy failure results in failure of the ATP-dependent  $\text{Na}^+/\text{K}^+$  pumps, massive  $\text{Na}^+$  and  $\text{H}_2\text{O}$  influx, cytotoxic oedema and neuronal cell death by necrosis [21]. These events propagate excitotoxicity and its consequences, and increase production of nitric oxide synthase (NOS) which results in a compensatory increase in cerebral blood flow [21]. In turn this triggers the production of potent RNS that actively contribute and further neuronal injury. As discussed above, the intracellular accumulation of  $\text{Ca}^{2+}$  is a key step in neuronal cell death. Mitochondria are the key intracellular  $\text{Ca}^{2+}$  buffers to maintain intracellular homeostasis [52]. Following intracellular  $\text{Ca}^{2+}$  accumulation, cell death is mediated by several mitochondrial enzymes that induce apoptosis.

### Circadian rhythms, the innate immune system, and neonatal encephalopathy

Circadian rhythms (CRs) are a wide range of physical, behavioural, and social rhythms that fluctuate in an approximately a 24-hour period. CRs are present in almost every living organism and are thought to provide an evolutionary advantage [55] by enhancing metabolic efficiency [56] and host defence at times of greater risk of pathogen exposure [57]. Nearly every human physiological function, including the immune and metabolic systems, are under circadian influence and many pathologic events demonstrate time-of-day patterns [58] (Figure 6). Zeitgebers, CR entrainment factors, such as light exposure are the environmental cues that synchronise internal circadian clocks to the external environment. The central circadian pacemaker is the suprachiasmatic nucleus (SCN) in the hypothalamus which is entrained to the external environment by light detected

by intrinsically photosensitive retinal ganglion cells in the retina, and transmitted to the SCN by the retinohypothalamic tract [59]. Output factors from the SCN which synchronise peripheral clocks in every other tissue to the central circadian rhythm include autonomic innervation of peripheral tissues, endocrine signalling, and temperature regulation [60]. Although the SCN regulates peripheral clock oscillators, they remain autonomous and peripheral clocks can become desynchronised from the central oscillator [60]. This can happen when different zeitgebers come into conflict, such as light exposure and food intake, another potent zeitgeber of peripheral tissues. For example, simulated night-shift work has demonstrated desynchrony in rhythmic immune functions [61]. This chronodisruption has many implications for health and disease [62].

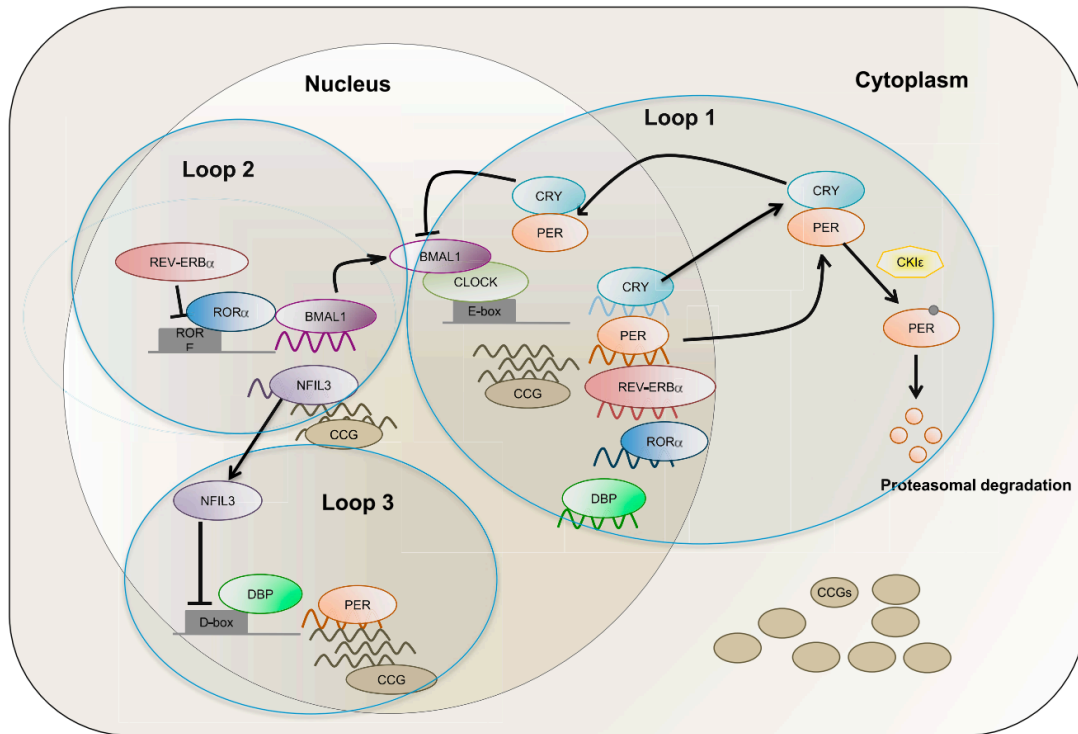




**Figure 6 Circadian networks and geophysical time, Allada et al, *New England Journal of Medicine*, 2021 [58].** The molecular circuitry of circadian clocks is encoded by an autoregulatory 24-hour transcription loop in the brain, where the clocks align sleep–wake and feeding cycles with the rotation of Earth on its axis. Clocks are also present in nearly all tissues of the body, composing a network of timekeepers that anticipate varying environmental conditions each day. Having evolved across all kingdoms of life, the molecular circuitry provides photosensitive species with a mechanism to enhance bioenergetic cycles and ensure escape from DNA-damaging effects of sunlight. BMAL1 denotes brain and muscle Arnt-like protein 1, CLOCK circadian locomotor output cycles kaput, CRY cryptochrome, PER period, RORE retinoic acid–related orphan receptor (ROR) response elements, and SCN suprachiasmatic nucleus.

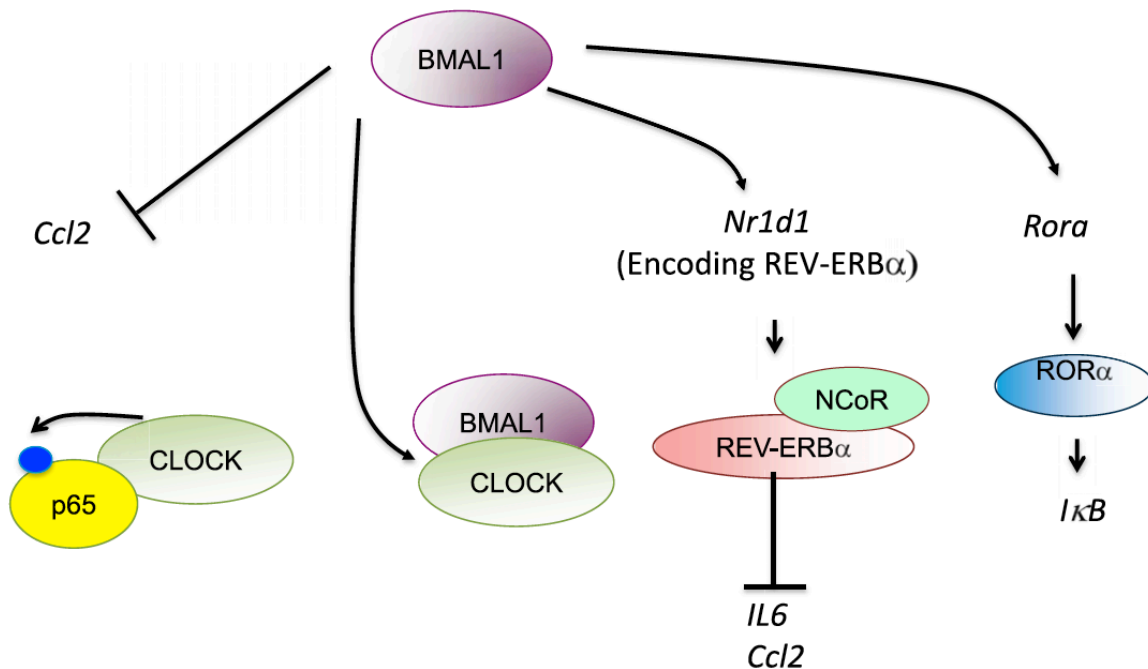
Fundamentally CRs occur at a subcellular level and are made up of circadian rhythm genes (CRGs). CRGs are present in almost every human cell and are entrained to the external environment and thus the central CR by the SCN as described above. The significance of the effect of circadian influence on human physiology was recognised by the 2017 Nobel prize in Physiology and Medicine being awarded to three scientists for their discoveries of the molecular mechanisms that control CRs. Up to 80% of protein-coding genes display diurnal cycling, making the CR the largest physiological regulatory network [63]. These genes form 3 interlocking transcription-translation feedback loops (TTFLs) (Figure 7). These CRGs are present in all immune cells [64] and play a key role in several aspects of innate immunity such as immune cell migration and functions such as cytokine production and phagocytic behaviour [65]. Basic Helix-Loop-Helix ARNT Like 1 (BMAL1) is the central mediator of circadian control of immune function, and it promotes an anti-inflammatory state [66] (Figure 8). Circadian locomotor output cycles kaput (CLOCK), cryptochrome (CRY), and REV-ERB $\alpha$  are other key circadian genes that regulate the expression of BMAL1 and also have a major role in the regulation of the immune system [67]. Circadian regulation of the innate immune system provides an anticipatory mechanism to coordinate response to the external environment [68]. However, circadian disruption is associated with worse innate immune response and outcome following hypoxic injury [69]. Circadian disruption has also been associated with increased risk of inflammatory disorders such as inflammatory bowel disease, type 2 diabetes mellitus, and an increased risk of cancer [70]. Improved understanding of the effect of CRGs on physiological process has a variety of medical implications including improved entrainment of CRs, improved timing of

drug and vaccine administration, and drugs that specifically target CRG transcription [71, 72].



**Figure 7 Molecular clock interlocking feedback loops, Curtis et al, Immunity, 2014 [73] .**

Loop 1 consists of the core clock proteins BMAL1 and CLOCK binding to E-box elements within the genes encoding the repressor proteins PER, CRY, REV-ERBa, RORa and DBP. After a period of time, PER and CRY can translocate back into the nucleus and repress their own expression by interfering with the BMAL1:CLOCK complex on the gene promoter. The expression of these proteins is regulated further by posttranslational modifications such as phosphorylation of PER by CKIε, which marks it for proteasomal degradation. Loop 2 consists of the alternate regulation by REV-ERBa and RORa on RORE promoter elements, which includes Bmal1 and Nfil3. Loop 3 consists of the alternate regulation by NFIL3 and DBP on D box promoter elements. The clock products from each of these loops can shuttle back to the nucleus and either repress or reactivate these loops. The transcription factors within each of these loops can also regulate clock-controlled genes (CCGs). These are genes that have a circadian profile of expression but do not feedback to affect the core molecular clock. If some of these CCGs are themselves transcription factors, they may confer a circadian profile of expression on their target genes. Therefore, the core clock components and CCGs have the capacity to regulate transcription of a wide variety of cellular programs that is independent of their function within the molecular clock.



**Figure 8** *BMAL1 is the central regulator of circadian control of the immune system, Curtis, Immunity, 2014 [73].* BMAL1 directly represses *Ccl2* expression, leading to lower numbers of the Ly6Chi inflammatory monocytes in circulation and lower recruitment of these inflammatory monocytes into inflamed tissues. BMAL1 sequesters CLOCK and prevents it from acetylating and activating p65, which leads to lower amounts of transcriptionally active NF- $\kappa$ B, leading to less induction of specific genes such as cytokines and regulators of survival and proliferation. BMAL1 drives the expression of *Nr1d1* (encoding REV-ERB $\alpha$ ) that can inhibit *IL6* and *Ccl2* expression. BMAL1 drives the expression of *Rora* that can increase the expression of *I $\kappa$ B*, a major negative regulator of NF- $\kappa$ B. This would have the effect of retarding the NF- $\kappa$ B complex as a nonactive form in the cytoplasm, preventing it from translocating into the nucleus and activating a range of genes including cytokines.

Sleep is the primary circadian behaviour and sleep wake cycles are intertwined with circadian rhythms. However recent changes to the environment, such as artificial lighting, can lead to disorganisation of the circadian rhythm and sleep cycles. Sleep disruption has major implications for health and disease, in particular for inflammatory and metabolic

conditions [74, 75]. Although sleep has traditionally been a neglected aspect of medicine, it's importance in health and disease is being increasingly recognised [76].

Melatonin is a chronobiotic agent [77] which is secreted by the pineal gland under the influence of the SCN in response to darkness [78]. Melatonin itself controls the central CR by directly influencing the SCN [79]. Melatonin interferes with the circadian gene TTFLs through the ubiquitin-proteasome system and increases the expression of BMAL1 to increase cellular survival [80]. Melatonin administration has been demonstrated to repair circadian clock gene disruption and normalise innate immunity and mitochondrial homeostasis [81].

Neonatal circadian rhythms remain poorly understood. CRs have been demonstrated in fetal life [82] however they are coupled to the maternal circadian rhythm and are entrained by maternal hormone exposure including melatonin, glucocorticoids, and dopamine [83]. Experimental models have demonstrated that lesioning the maternal SCN prevents entrainment of the fetal CR [84]. The neonatal CRs develop over the first few weeks and months of postnatal life [85], however it remains unclear how CRs are entrained in newborns and how external factors influence this entrainment [86]. Light is the only effective zeitgeber to be supported by evidence currently [87]. It has been suggested that disruption of entrainment factors in the postnatal period may have long-term health consequences [88]. Night-time delivery has been associated with increased risk of NE and worse neonatal mortality [89].

While the importance of CR entrainment is evident, many of the likely zeitgebers in neonatal life are disrupted in the NICU environment, including light exposure, regular feeding patterns, and exposure to melatonin in breastmilk [90]. Although sleep does not demonstrate a circadian pattern until around 10-12 weeks of life, sleep-wake cycles have been demonstrated within the first 12 hours of life [91] and longer duration to sleep onset is associated with worse outcome in NE [92]. It has been hypothesised that circadian and sleep disruption may contribute to further dysregulated inflammation and worse outcomes in neonatal encephalopathy. Potential zeitgebers are amenable to improved management and have demonstrated improved outcomes in other neonatal populations [93] and there is increasing recognition of the importance of these factors in the NICU [94, 95].

### Role of Cytokines in Neonatal Encephalopathy

Cytokines are polypeptides secreted by leukocytes and other immune cells that act primarily on haematopoietic cells to regulate immune and inflammatory reactions [96]. The definition is broad and there is some overlap with the classification of hormones, although cytokines are much more potent and are produced by many cell types as opposed to a single cell or tissue [97]. There are many different types of cytokines, and they are frequently classified into families according to the type of receptor to which they bind or by their function [98]. Cytokines have pleiotropic effects as they can bind to different receptors. Therefore, cytokines have context-dependent roles which affects their production and interaction with other cytokines and their receptors [99]. Serum cytokines play a crucial role in the pathogenesis of NE [100-106]. Dynamic changes in serum cytokines

have been associated with improved outcomes in trials of therapeutic hypothermia (TH) for infants with NE [107]. Melatonin can inhibit or stimulate both pro- and anti-inflammatory cytokines depending on the clinical context by activation of the Nucleotide-binding and oligomerization domain (NOD)-, leucine-rich repeat domain (LRR)- and NOD-like receptor protein 3 (NLRP3) inflammasome, by inhibition of Nuclear Factor kappa B (NF- $\kappa$ B), and by regulating the production of reactive oxygen species (ROS) and anti-oxidant enzymes [108]. Diurnal variation in cytokine production has also been demonstrated with peak production of pro-inflammatory cytokines occurring at night and in the early morning [109].

#### *IL-1 receptor family cytokines*

Two pro-inflammatory cytokines that are crucial to the inflammatory response on the innate immune system, IL-1 $\beta$  and IL-18, are dependent on the formation of inflammasomes for their secretion. NLRP3 is the most widely characterised inflammasome upon which the secretion of IL-1 $\beta$  and IL-18 is dependent [110]. Inflammasome activation requires 2 signals from several damage or pathogen associated molecular patterns (DAMPs or PAMPs) which activate pathogen recognition receptors (PRRs) to form intracellular inflammasomes [111]. Inflammasome activation results in cleavage of pro-IL-1 $\beta$  and pro-IL-18 to their mature forms by caspase-1 [112]. IL-1 $\beta$  is produced by a variety of cells of the innate immune system including neutrophils, dendritic cells and epithelial cells, but its secretion has best been characterised in macrophages and monocytes [113]. IL-1 $\beta$  and IL-18 play a key role in host defence and inflammation [114]. IL-1 $\beta$  binds to receptors IL-1R1 and IL-1R2 which are expressed on a number of immune cells including neutrophils, T-

lymphocytes, fibroblasts, epithelial cells, endothelial cells, and hematopoietic cells [115]. Binding of IL-1 $\beta$  produces a wide range of pro-inflammatory responses including leukocyte recruitment and survival, production of acute phase reactants, and promotes TH17 differentiation [116]. The pro-inflammatory effects of IL-18 is much less characterised than IL-1 $\beta$ . IL-18 increases cell adhesion molecules, production of nitric oxide synthase, chemokine production and it is involved in the TH1 response by modulating production of IFN- $\gamma$ . However the effects of IL-18 are not as significant as IL-1 $\beta$  [117]. Infants with HIE demonstrated elevated serum concentrations of IL-1 $\beta$  compared to controls [118]. While some studies have shown an association between IL-1 $\beta$  and adverse findings on MRI brain [101], these findings are not consistent across all studies [119].

IL-1 receptor antagonist (IL-1RA) is produced by the same cells as IL-1 $\beta$ , particularly monocytes, macrophages, neutrophils, and dendritic cells [120]. IL-1RA binds to the same receptors as IL-1 $\beta$ , IL-1R1 and IL-1R2, but interacts differently thus the co-receptor IL-1RAcP is not recruited and no signal is transduced [121, 122]. Therefore, IL-1RA competitively inhibits IL-1 $\beta$ . IL-1RA also inhibits the pathogenic activity of the NLRP3 inflammasome [123]. Anakinra is the most commonly used recombinant IL-1RA (rIL-1RA) currently in use to treat inflammatory conditions including rheumatoid arthritis. Despite the promise of IL-1RA as a therapeutic target for NE, when given in combination with TH, rIL-1RA accumulated within the CNS and paradoxically upregulated the IL-1 pathway in a model of NE [124].



### Type 1 (haematopoietin) receptor family cytokines

Type 1 (haematopoietin) receptor cytokines including IL-2, IL-6, EPO and GM-CSF have been associated with adverse outcome in NE. IL-2 is primarily produced by antigen-activated T cells [125]. It's role in the inflammatory process is to regulate the differentiation and proliferation of pro- and anti-inflammatory T cells, and to regulate T cell metabolic programming [126]. Elevated IL-2 in the first 24 hours of life in infants with HIE has been associated with adverse outcome [101], and elevated IL-2 persists into childhood in infants with NE [23].

IL-6 is a crucial regulator of immune response, inflammation, and haematopoiesis [127]. It is primarily produced by monocytes and macrophages in response to tissue injury or infection and it plays an important role in host defence. However, excessive or persistent IL-6 production leads to the uncontrolled inflammatory response cytokine storm, and it has a pathological role in the development of inflammatory diseases [128]. Pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  interact with transcription factors NF- $\kappa$ B and signal transducer and activator of transcription 3 (STAT3) to induce hyperactivation of NF- $\kappa$ B, leading to the production of pro-inflammatory cytokines including IL-6. As NF- $\kappa$ B is a target of IL-6, activation of NF- $\kappa$ B and STAT3 leads to positive feedback loop of NF- $\kappa$ B activation by IL-6–STAT3 axis, known as the IL-6 amplifier, a key regulator of the local initiation model of inflammation [129]. IL-6 promotes the proliferation and activation of T cells, differentiation of B cells, and regulates many acute phase reactants in the liver including CRP and albumin [130]. Due to its importance in inflammatory diseases IL-6 blockade is an important immunotherapeutic strategy and specific IL-6 blockade with humanized anti-IL-6 receptor

antibody, tocilizumab, is an effective therapy in many inflammatory conditions [131]. IL-6 is elevated in infants with NE compared to controls [100, 118], and elevated IL-6 is associated with adverse findings on MRI brain [101] and adverse motor and cognitive outcomes [100] in infants with NE.

Erythropoietin (Epo) is a powerful pleiotropic cytokine with many haematopoietic and non-haematopoietic functions [132]. Of all the performance enhancing drugs taken by Lance Armstrong, he described Epo supplementation as 'high octane rocket fuel'. In response to systemic hypoxia, hypoxia-inducible factors (HIFs) stimulate the liver and kidneys to increase production of Epo [133] and increase expression of its receptor Epo-R [134]. Epo stimulates erythropoiesis in bone marrow, however many other cells, including a variety of cell types in the brain express Epo-R [135] where Epo has many non-haematopoietic functions including angiogenesis, neurogenesis, and oligodendrogenesis. Following ischaemic injury Epo limits damage to surrounding tissue by limiting the destructive potential of TNF- $\alpha$  and other pro-inflammatory cytokines [136] and protects cells from apoptosis [137]. Due to its promising therapeutic potential [138], recombinant Epo (rEPO) as an adjunct therapy to TH has been under investigation. A systematic review identified 6 RCTs of rEPO in NE [139] however only one phase II trial compared rEpo vs placebo as an adjunct to HT and no difference was found in the risk of death but the rEPO & TH group had reduced brain injury on MRI and improved motor outcomes at 12 months [140]. In a major blow to the promise of Epo in NE, the largest phase III trial of Epo for NE, the HEAL trial, recently reported no reduction in mortality or neurodevelopmental impairment in those treated with Epo compared to placebo, and a higher rate of serious

adverse events in those treated with Epo [141]. The phase III PAEAN trial is currently in progress [142].

GM-CSF is another cytokine with haematopoietic and immune modulating functions. It is produced primarily by T helper cells, but macrophages, endothelial cells and fibroblasts also have a role [143]. Although the initial function of GM-CSF was identified as a haematopoietic growth factor, it is now recognised that the majority of myeloid cells do not require GM-CSF under steady state conditions for myelopoiesis [144]. Instead, under inflammatory conditions, GM-CSF is recognised as a signalling molecule between tissue-invading lymphocytes and myeloid cells. GM-CSF promotes inflammation by the activation of phagocytes and the release of ROS, and recruitment of neutrophils and monocytes into the CNS, and their differentiation into pathogenic effectors through inflammasome activation [145]. While monocyte derived macrophages appear to promote inflammation in the CNS, GM-CSF may also have a protective role by promoting production of microglia derived macrophages which clear debris and suppress cellular metabolism [146]. GM-CSF is lower in infants with NE and abnormal MRI and compared to those with normal MRI brain [104] however it was elevated at school age in children with NE compared to controls [23].

#### *Type 2 (interferon) receptor family & tumour necrosis factor family cytokines*

Interferon-gamma (IFN- $\gamma$ ) is a crucial cytokine in the regulation of immune and inflammatory responses. It is primarily produced by natural killer cells, innate lymphoid cells, and adaptive immune cells such as TH1 cells and CD8+ cytotoxic T lymphocytes [147]. IFN- $\gamma$  receptors are expressed on nearly all immune cells including macrophages. IFN- $\gamma$  was

originally identified as a macrophage activating factor, and they are a major target of IFN- $\gamma$  [147]. During infection or tissue damage IFN- $\gamma$  production is induced by cytokines IL-12 or IL-18, or activation of PRRs [148]. IL-4, IL-10, transforming growth factor- $\beta$  (TGF- $\beta$ ), and glucocorticoids inhibit production of IFN- $\gamma$ . Binding of IFN- $\gamma$  activates transcription of hundreds of interferon-stimulated genes (ISGs) that promote a range of pro-inflammatory effects including production of pro-inflammatory cytokines including IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , resistance to anti-inflammatory cytokines including IL-10, increased production of ROS and phagocytic receptors, and increased production of leukocyte chemokines [148]. Elevated serum IFN- $\gamma$  is associated with abnormal neurological outcome in infants with neonatal HIE [102].

IL-10 is a cytokine with potent anti-inflammatory properties. During infection it limits damage to the host by limiting the immune response to invading pathogens [149]. It is produced by many cells, the most important of which are monocytes, macrophages, Tregs, T helper (TH)2, and other CD4+ T cells [150]. IL-10 expression is controlled by IFN- $\gamma$  and IL-10 itself by positive or negative feedback on its own expression. Macrophages are the main target of IL-10 where it maintains mitochondrial integrity preventing the release of ROS and inhibits inflammasome activation and the release of pro-inflammatory cytokines [151]. IL-10 is higher in infants with NE compared to controls [100]. Elevated IL-10 is a reliable prognostic biomarker for grade of severity of NE, neonatal mortality, and adverse early childhood outcome [101, 152].

Another pro-inflammatory cytokine critical to the regulation of the innate immune response to infection or tissue damage is TNF- $\alpha$ . It is primarily produced by monocytes and

macrophages, but neutrophils, NK cells, mast cell and B and T lymphocytes also have a role [153]. In the CNS TNF- $\alpha$  is mainly produced by microglia, astrocytes and neurons [154]. The functions of TNF- $\alpha$  are mediated by 2 receptors, TNFR1, expressed on all human cells, and TNFR2, expressed on primarily on immune cells, neurons, and endothelial cells [155]. TNFR1 expresses an intracellular death domain that recruits proteins that result in cell death. TNF- $\alpha$  plays an important role in host defence against infection, resolution of inflammation, tissue regeneration and inhibition of tumour growth [156] but its dysregulation characterises many inflammatory pathologies. TNF- $\alpha$  is a pleiotropic cytokine with many pro-inflammatory effects including disruption of the macro and microvascular circulation, contributing to vasodilation and oedema formation, induction of ROS production and worsening oxidative stress, increasing epithelial leukocyte adhesion molecule expression and recruitment of immune cells, increases production of other pro-inflammatory cytokines, and initiates the coagulation cascade [157, 158]. Due to the importance of TNF- $\alpha$  signalling in chronic inflammatory disorders, several TNF- $\alpha$  inhibitors have been developed successfully. Etanercept, infliximab, and adalimumab are TNF- $\alpha$  inhibitors currently used in conditions including Crohn's disease, rheumatoid arthritis and ankylosing spondylitis [156]. TNF- $\alpha$  is increased in infants with NE compared to controls [118], and in NE elevated TNF- $\alpha$  associated with increased severity of brain injury on MRI [159].

Although the inflammatory role of TNF- $\alpha$  is well understood much less is known about the other TNF superfamily member, TNF- $\beta$ , also known as lymphotoxin- $\alpha$ . TNF- $\beta$  is secreted by B and T lymphocytes, and NK cells [160]. It binds with high-affinity to the same receptors as TNF- $\alpha$ , TNFR1 and TNFR2 [161], and produces similar pro-inflammatory effects.

However, while TNF- $\alpha$  is expressed in both soluble and transmembrane forms, TNF- $\beta$  lacks the transmembrane domain and therefore its cell signalling effects are much more limited [162]. TNF- $\beta$  remains elevated at school age in children with NE compared to controls [23].

#### CXC chemokine family and vascular endothelial growth factor cytokines

IL-8 is a chemokine primarily produced by monocytes and endothelial cells, and its synthesis is stimulated by cytokines IL-1 $\beta$  and TNF- $\alpha$  [163]. It binds to receptors on many cell types including neutrophils, monocytes, astrocytes, microglia, and endothelial cells [164] and is known to promote pro-inflammatory activities including immune cell activation and angiogenesis. IL-8 is primarily known as a neutrophil chemoattractant [165] and increases neutrophil adhesion molecules expression, neutrophil activation, and enhances the metabolism of reactive oxygen species (ROS) [166]. However, IL-8 also possesses anti-inflammatory properties by preventing adhesion of leukocytes to activated endothelial cells [167]. Elevated serum IL-8 in the first 2 days of life is associated with worse grade of encephalopathy, higher risk of mortality [104], and higher risk of abnormal neurodevelopmental outcome [168]. IL-8 is higher at school age in children with NE compared to controls [23].

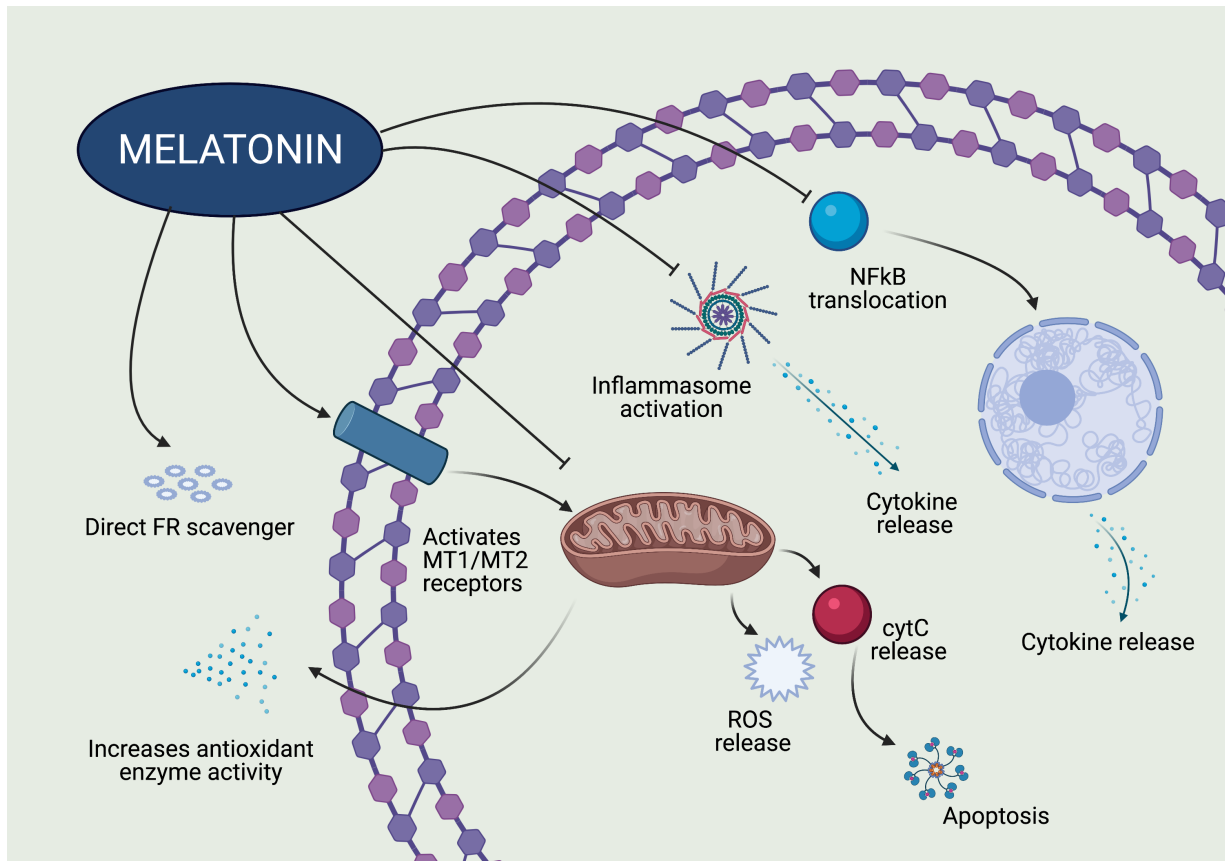
VEGF is a family of cytokines, the best known of which is VEGF-A, from here on referred to as VEGF. It is a critical cytokine during tissue repair following inflammation. VEGF is another cytokine whose expression is stimulated by HIF in response to systemic hypoxia [169]. It is primarily produced by macrophages but nearly all immune cell types and endothelial cells secrete VEGF [170]. Its production is regulated by other inflammatory

cytokines including IL-10 and IFN- $\gamma$  [171]. VEGF binds to VEGF receptors VEGFR-1,-2,-3 and neuropilin (NRP) family members NRP-1 and -2. It is best known for its role in angiogenesis and as the master regulator of CNS blood vessel formation [172]. VEGF promotes new blood vessel formation by stimulating endothelial cell proliferation and migration [172]. The formation of new blood vessels provides improved brain perfusion however it also leads to increased permeability of the blood brain barrier and vessel leakage [173]. VEGF also results in vascular haemostasis [170], monocyte recruitment [174], haematopoiesis, and survival of cells expressing VEGFR and NRP receptors including neuronal cells neurons, astrocytes, microglia, and oligodendrocytes [175]. Elevated VEGF was found in cord blood of term neonates with perinatal asphyxia [176], however decreased VEGF on day 1 of life was associated with increased mortality in NE [177].

### Melatonin treatment in neonatal encephalopathy

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone which is primarily produced by the pineal gland in the brain [78]. It is synthesized initially by the conversion of tryptophan to serotonin, then to N-acetylserotonin, and finally to melatonin (or N-acetyl-5-methoxytryptamine) [178]. The release of melatonin is regulated by intrinsically photosensitive retinal ganglion cells in the eye which express the unique photosensitive pigment melanopsin and detect changes in light exposure [179]. As a result there is significant 24-hour variation in blood melatonin levels, with concentrations being lower in the day and higher at night correlating with light exposure [180]. While melatonin has an important role as a chronobiotic agent, regulating the circadian rhythm, it also has

anti-inflammatory [181], anti-oxidant [182], and anti-apoptotic [183] properties which make it a promising intervention for neuroprotection in NE (Figure 9).



**Figure 9 Proposed mechanisms of action of melatonin in neonatal encephalopathy.** Melatonin exerts anti-inflammatory effects by inhibiting nuclear factor  $\kappa$  B (NFKB) activation preventing the release of pro-inflammatory cytokines, and by inhibiting NLRP3 inflammasome activation, again preventing the release of pro-inflammatory cytokines. Melatonin acts as an anti-oxidant by inhibiting reactive oxygen species (ROS) production from the mitochondria, by stimulating melatonin receptors MT1 and MT2 to produce antioxidant enzymes, and by acting as a direct free radical scavenger. Melatonin acts as an anti-apoptotic agent by preventing cytochrome C (cytC) release from the mitochondria. Created using Biorender.



Melatonin is a potent context-specific immunomodulator and can stimulate or suppress the inflammatory response [108]. It easily crosses the blood brain barrier (BBB) and high levels accumulate in the central nervous system [184]. Under conditions of excessive immune responses melatonin acts as an anti-inflammatory molecule [185]. It exerts its influence on the innate immune system by regulating the lifespan of leukocytes by interfering with processes of cell death, regulating the release of pro-inflammatory cytokines and leukotrienes, and modulating the production of pro inflammatory enzymes [186]. Recent studies have shown that increased neutrophil activation [44] and elevated circulating pro-inflammatory cytokines [105] are associated with worse outcomes in NE. Clinical trials have demonstrated a reduction in circulating leukocytes and pro-inflammatory chemokines in patients treated with TH [107], suggesting that modulation of brain injury in NE occurs via immunosuppression. Further studies have demonstrated that administration of melatonin leads to a downregulation of neutrophil activity and pro-inflammatory cytokines in the neonatal population [187-189]. Melatonin also has potent anti-oxidant properties as it stimulates the production of anti-oxidant enzymes, including superoxide dismutase, glutathione peroxidase, and glutathione reductase, through melatonin receptors MT1 and MT2, by inhibiting the production of the pro-oxidant enzyme nitric oxide synthase [182], and as a direct free radical scavenger, reducing the toxic effects of reactive oxygen species and leading to further reduction in oxidative stress [190]. Newborns are particularly vulnerable to oxidative stress [191], and oxidative stress is a critical contributor to brain injury in NE. Newborns with NE are unable to compensate for the excess production of reactive oxygen species following hypoxic or ischaemic insult. This in turn leads to an

accumulation of toxins and further systemic inflammation which results in worsening brain injury. Studies of several conditions involving oxidative stress in newborns, including perinatal asphyxia, sepsis, and respiratory distress syndrome, have demonstrated a reduction in oxidative stress with melatonin supplementation [192]. Melatonin also regulates apoptotic pathways by interfering with caspase-dependent (cytochrome C) and caspase-independent (apoptosis-inducing factor) pathways of mitochondrial cell death [183]. These effects are context specific, and melatonin can enhance or suppress these apoptotic effects [193]. In NE, oxidative stress combines with mitochondrial dysfunction to result in mitochondrial energy failure, a key regulator of apoptotic cell death [52]. Melatonin protects against neuron apoptosis via a wide range of effects, including reduced release of cytochrome c, increased expression of anti-apoptotic proteins, and reduced expression of pro-apoptotic proteins. These actions help prevent excess mitochondrial permeability and stabilise the mitochondrial membrane potential [194], leading to reduced apoptotic cell death.

For these reasons melatonin has been proposed as an adjunctive treatment to therapeutic hypothermia in infants with NE [181]. Several animal models of NE have demonstrated improved outcomes with melatonin supplementation [195-197]. Melatonin has been studied in several other conditions in the neonatal population and has an excellent safety profile in the neonatal population [192, 198]. Although melatonin is a promising neuroprotective therapy for infants with NE, progress on its potential implementation has been slow to date [199].

## Definitions of neonatal encephalopathy

It is an ongoing conundrum what the correct terminology, definition, and diagnostic criteria are for the condition of neonatal encephalopathy (NE) [9]. Many have advocated for the use of the term NE [8, 9, 200], however there are some strong and significant dissenting opinions on this matter [201]. The most significant effort to date to resolve this matter has been undertaken by the American College of Obstetrics and Gynaecology (ACOG) & American Academy of Pediatrics (AAP) Taskforce on Neonatal Encephalopathy [1]. Most understand NE to be a clinically defined syndrome of disturbed neurological function in the first few days of life, manifested by reduced level of consciousness, difficulty initiating or maintaining respiration, or seizures in infants born at or greater than 35 weeks gestational age. This clinical definition is very broad and does not imply causation, unlike the term hypoxic ischaemic encephalopathy (HIE), which many have suggested should be reserved for a cause-specific subset of those with NE. Multiple aetiologies of NE have been identified [202], and HIE is thought to be a contributing factor in 29% of cases of NE and a stand-alone factor in only 4% [203]. A further difficulty in the diagnosis of NE is the time restriction required for the initiation of therapeutic hypothermia (TH), which requires initiation within 6 hours of birth [204]. The ACOG/AAP Taskforce on NE outlines criteria to determine whether of NE may be a result of HIE, however many of these tests require substantially more time than 6 hours to complete [1]. As mentioned above, the lack of consensus regarding the terminology and definition of NE results in difficulty estimating the incidence and prevalence of the condition and makes comparisons of outcomes across groups more challenging. In the absence of a universal definition or diagnostic criteria, many use the

eligibility criteria for randomised controlled trials of TH to identify those with the condition [205]. Enrolment to trials of TH was restricted to those with moderate-severe NE, and many different neurological assessments were used to categorise the severity of NE. As a result, many studies do not represent the full spectrum of the condition and many with mild NE are not included in trials of therapeutic interventions or in studies of the epidemiology of the condition [206]. To follow a more inclusive process for consensus building for a definition and diagnostic criteria for NE, a comprehensive analysis of the currently used terminology, definitions, and diagnostic criteria is required.

#### Prognostic factors in neonatal encephalopathy

NE is associated with high rates of mortality and long-term neurodisability, despite routine use of TH in high resource settings [204]. However predicting outcomes for individual infants with NE remains a significant challenge [207]. Many different markers have been evaluated for their prognostic value in NE, including clinical examination [208], blood and cerebrospinal markers [106], neurophysiological [209], and radiological markers [210]. There are benefits to each type of prognostic biomarker, however no tool has proven superior to all others nor to provide very consistent and highly accurate prognosis [211] but MRI, and MRS in particular, has emerged as a very promising methodology in recent years [212]. The significance of early recognition of those at risk of adverse outcome is becoming increasingly evident [213]. Improved understanding of injury progression in NE means there is a longer therapeutic window for treatments, therefore higher significance of identifying those at risk and implementation of the neuroprotective strategies [214].

## Hypothesis

We hypothesised that CRs are disrupted in the early stages of NE and is related to more dysregulated inflammation and worse outcomes. We hypothesised that melatonin regulates the systemic immune response in infants with TH for NE.

## Aims

**Aim 1. Examine the effect of melatonin on the innate immune system in patients with NE**

### Rationale

Melatonin is a potent immune-modulating agent and may provide neuroprotection as an adjunctive therapy to therapeutic hypothermia.

### Objectives

To examine differences immune response in neutrophil and monocyte phenotype, inflammasome gene expression, and microRNA gene expression following melatonin treatment in infants with NE.

### Deliverables

Understand the immune-modulating effects of melatonin in NE, which may provide the basis for its implementation as an adjunctive therapy to therapeutic hypothermia.

Presentation at Joint European Neonatal Societies meeting 2021 and at Irish Neonatal Research Symposium 2021. Paper for publication is being prepared.

**Aim 2. Examine the effect of melatonin treatment on inflammatory cytokines in infants with NE and healthy controls**

Rationale

Melatonin alters inflammatory cytokine profile under conditions of dysregulated inflammation however it has a context-specific effects and it is unclear what the effects may be in infants with NE.

Objectives

Examine the effects of melatonin treatment on serum cytokines in infants with NE and healthy controls.

Deliverables

Presentation at Pediatric Academic Societies 2021 and the Neonatal Society UK 2021. Paper for publication is being prepared.

**Aim 3. Examine the current evidence for melatonin treatment in infants with NE**

Rationale

The level of evidence for routine use of melatonin as a monotherapy or adjunctive therapy to TH in NE is uncertain and synthesis of the currently available evidence is required.

### Objectives

Synthesise the current evidence from human randomised controlled trials of melatonin in infants with NE.

### Deliverables

Presentation at Joint European Neonatal Societies 2021. Cochrane review of melatonin treatment in NE has been submitted for editorial review.

## **Aim 4. Examine the current evidence for prognostic factors in NE**

### Rationale

Many radiological, biochemical, and neurophysiological markers have been proposed as valuable prognostic tools in NE however it remains uncertain which provide the most accurate prognostic value.

### Objectives

Examine and synthesise the current evidence for MRI, blood, and cerebrospinal fluid markers as prognostic tools in NE.

### Deliverables

Presentations at Joint European Neonatal Societies 2021 and European Academy of Pediatrics 2022. Papers for publication are being prepared.

## Chapter 2: Systematic review of terminology, definitions, and eligibility criteria in trials of NE

### Abstract

**Background:** Appropriate terminology and definitions of neonatal encephalopathy (NE), hypoxic ischaemic encephalopathy (HIE), and perinatal asphyxia (PA) have been debated for the past 20 years. Many, including the American Academy of Pediatrics, have advocated for NE to be used as it does not assume an aetiology, however this is not universally accepted. Moreover, there is no generally accepted case definition criteria for NE/HIE/PA. Instead, the criteria used in trials of therapeutic hypothermia (TH) are frequently employed. However, variation in inclusion and exclusion criteria lead to variation in participants recruited and the severity of NE/HIE. This may explain differences in mortality in control groups between trials. Consistent terminology and case definition criteria would clarify the clinical entity, standardise participant criteria in future trials, and provide greater generalisability of trial results. We completed a systematic review to examine the terminology, definitions, and case participant criteria used in trials of NE/HIE.

**Methods:** A comprehensive search was run in December 2019 in the databases Embase, MEDLINE, CENTRAL, CDSR, the WHO for randomized controlled trials (RCTs) of interventions for the treatment of NE/HIE/PA. Any definition or criteria for NE or HIE were eligible. Follow up studies of original trials were excluded. Outcomes for this study were a description of the terminology, definitions, and participant inclusion/exclusion criteria. 2 reviewers



independently screened the results of the literature search, and 2 reviewers independently extracted the data. The qualitative results were synthesised in a narrative summary.

**Results:** The literature search identified 4375 results, of which 62 were included in the qualitative synthesis. HIE was the most frequently used descriptive term (53/62), next PA (32/62), and NE was least frequent (15/62). Several studies used different terms interchangeably. Inclusion criteria were divided into perinatal asphyxia, of which Apgar scores (58/62) and evidence of acidosis (53/62) were the most frequently used. However, there was significant variation between timing and cut-off values in Apgar score, and cut-off score in pH or base excess. Evidence of neurologic dysfunction most frequently required reduced level of consciousness (LOC) (51/62), reduced tone (50/62), and abnormal reflexes (48/62) but seizures were less frequently required (38/62). The threshold age for exclusion varied frequently between studies and as did the exclusion of participants with mild NE/HIE (35/62).

**Discussion:** This review identified variation in terminology used, major points of agreement between studies in the requirement inclusion and exclusion criteria for trial participants, and major points of variance between studies. These results will inform a consensus process for developing a definition and case definition of NE/HIE.

## Introduction

Neonatal encephalopathy (NE) is a syndrome of disturbed neurological function in term or late preterm neonates in the first few days of life' [201], while hypoxic ischaemic encephalopathy (HIE) has been defined as a cause-specific subgroup of NE caused by inadequate blood flow and oxygen delivery to the brain [215]. Despite this distinction many use the term HIE in preference to NE to refer to the broader condition or use the terms interchangeably. Perinatal asphyxia refers to impaired gas exchange in the intrapartum or immediate postnatal period [1]. There is no specific diagnostic test for the condition, and it is diagnosed based on generally accepted clinical and biochemical findings. NE/HIE/PA has a multifactorial aetiology [2] and is associated with multi-organ dysfunction [216]. Some argue that NE is an inadequate term for the condition [201]. It has been an ongoing debate for over 20 years what appropriate terminology to refer to newborns with brain injury and evidence of perinatal asphyxia and there is no conclusion or consensus on the issue to date [8, 9, 200, 217].

Although many infants have been exposed to perinatal asphyxia (PA), only a proportion of those with PA will have neurological dysfunction, and some will not have significant early signs of PA but will have significant neurological dysfunction [218]. Early identification of infants eligible for therapeutic hypothermia (TH) is essential within the first 6 hours of life as it is the gold standard treatment in high resource settings [4].

In the absence of consensus terminology or diagnostic criteria for NE, the case definition for NE has been implied from the eligibility criteria for trials of NE [219, 220]. While this provides some clarity on the diagnostic criteria, it results in a restrictive definition that does not include all patients with the condition nor all those at risk of adverse outcome. For example, RCT trials of TH were limited to those with moderate to severe NE/HIE and trial eligibility criteria reflect this. However, there is a spectrum of severity of NE/HIE and this definition excludes those with mild NE although it is evident that they are at increased risk of adverse outcome compared to the general population [221, 222]. There is controversy [223] about the use of TH for patients with mild NE, despite the lack of evidence for any benefit [224]. So, although patients with mild NE may not be eligible for TH there are at increased risk of adverse outcome and should be included in any definition of the condition and should be considered for future trials of therapies for NE/HIE. Furthermore, a direct comparison of the application of the eligibility criteria demonstrated significant difference in the number of infants eligible for TH depending on which neurological exam is used [205]. There is a need for standardisation of the criteria for TH and the neurological exam in particular [225], and more broadly for the inclusion of those diagnosed with mild NE.

This systematic review aimed to describe the frequency of terminology, definitions, and the eligibility criteria used for NE/HIE in clinical trials. The purpose was to identify similarities and differences between trials, how this may impact control group mortality between trials, and ultimately provide the background to develop consensus terminology,

definition, and eligibility criteria for trials of interventions for patients with NE. We hypothesised that there would be variation in terminology, definitions, and eligibility criteria to identify participants in trials for NE.

## Methods

This study was developed as an extension of a registered protocol with Prospero (CRD42020170265), a systematic review of reported outcomes in RCTs in NE. The only difference from the published protocol in this review is in search and screening methodologies. Systematic reviews were excluded in this review as the applicable eligibility criteria are for the inclusion of the study rather than for individual participants in RCTs. This systematic review was conducted in accordance with the published protocol and the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) reporting guideline [226].

### Information sources and search strategy

We systematically searched 5 major databases, Embase, MEDLINE, CENTRAL, CDSR, and WHO in December 2019 to identify all relevant studies published between January 1966 and December 2019. Additional data sources were identified through screening of all relevant primary studies and review articles, and by discussion with experts in this area. Only papers available in English were included. We used MESH and search terms to identify RCTs in neonatal encephalopathy, hypoxic ischaemic encephalopathy, or perinatal asphyxia. The completed search strategy and full search terms are available in Supplementary Methods 1. Authors were contacted for additional details not included in the publication if they were required for inclusion.

## Selection Criteria

Only RCTs (or protocols for RCTs) of interventions in NE in last 20 years were eligible for inclusion in this systematic review. Any definition of NE/HIE/PA that included features of perinatal asphyxia or encephalopathy was acceptable. Any intervention for NE was acceptable for inclusion. Studies that included participants <34 weeks GA were excluded. Studies of other design types such as case reports, case series, in vitro studies, or animal studies were excluded. Studies that reported clinical or biochemical eligibility criteria for diagnosis or inclusion in the RCT were extracted and included in the qualitative analysis.

## Outcome definition

The primary outcome was to describe the different terminology and definitions used for the condition, and to describe the types and frequency of different participant eligibility criteria for trials of treatments for NE/HIE.

## Screening and Selection Process

The results of the literature search were screened for inclusion against the selection criteria above by 2 reviewers independently (FQ, DD), initially by screening titles and abstracts and subsequently screening the full text of remaining studies. Disagreements were resolved by discussion (FQ, DD). No quality assessment was completed as the purpose of the study was to describe variation in terminology, definitions, and eligibility criteria in RCTs for NE and no meta-analysis was intended or performed.

## Data Extraction

A data extraction form (DEF) was developed (TH, EM) and piloted on 10 randomly selected studies. The DEF included the following domains: study descriptive data, terminology and definitions used, eligibility criteria employed, and study outcomes. Eligibility criteria were categorised as inclusion and exclusion criteria. Data from all included studies were extracted by 2 investigators independently (TH, AB, GK) and disagreements were resolved by consensus prior to data synthesis.

## Data Synthesis

A meta-analysis was not proposed or conducted as part of this systematic review. Categorical data was synthesised and presented as counts and percentages. Grouping data into perinatal asphyxia/neurological assessment categories was decided prior to data extraction. Where descriptive details were extracted from individual studies this is presented directly. All quantitative analyses were conducted using SPSS software.

## Results

The literature search yielded 4394 results and 10 further records were identified through a search of the grey literature and the reference lists of retrieved articles. 4379 results remained following the exclusion of duplicates. We excluded 3952 records during title and abstract screening leaving 243 for full text review. Of these, 62 studies were included in the qualitative synthesis (Figure 10).

RCTs were conducted in 20 different countries and published from 1998 to 2020. In total 5148 participants were included in the 62 included studies. The effect of 14 different interventions including whole body TH, selective head cooling, TH plus inhaled xenon, EPO, darbepoetin, melatonin, allopurinol, ascorbic acid and oral ibuprofen, magnesium sulfate, phenobarbital, and pyritinol were examined in RCTs. All studies were conducted in a hospital setting.



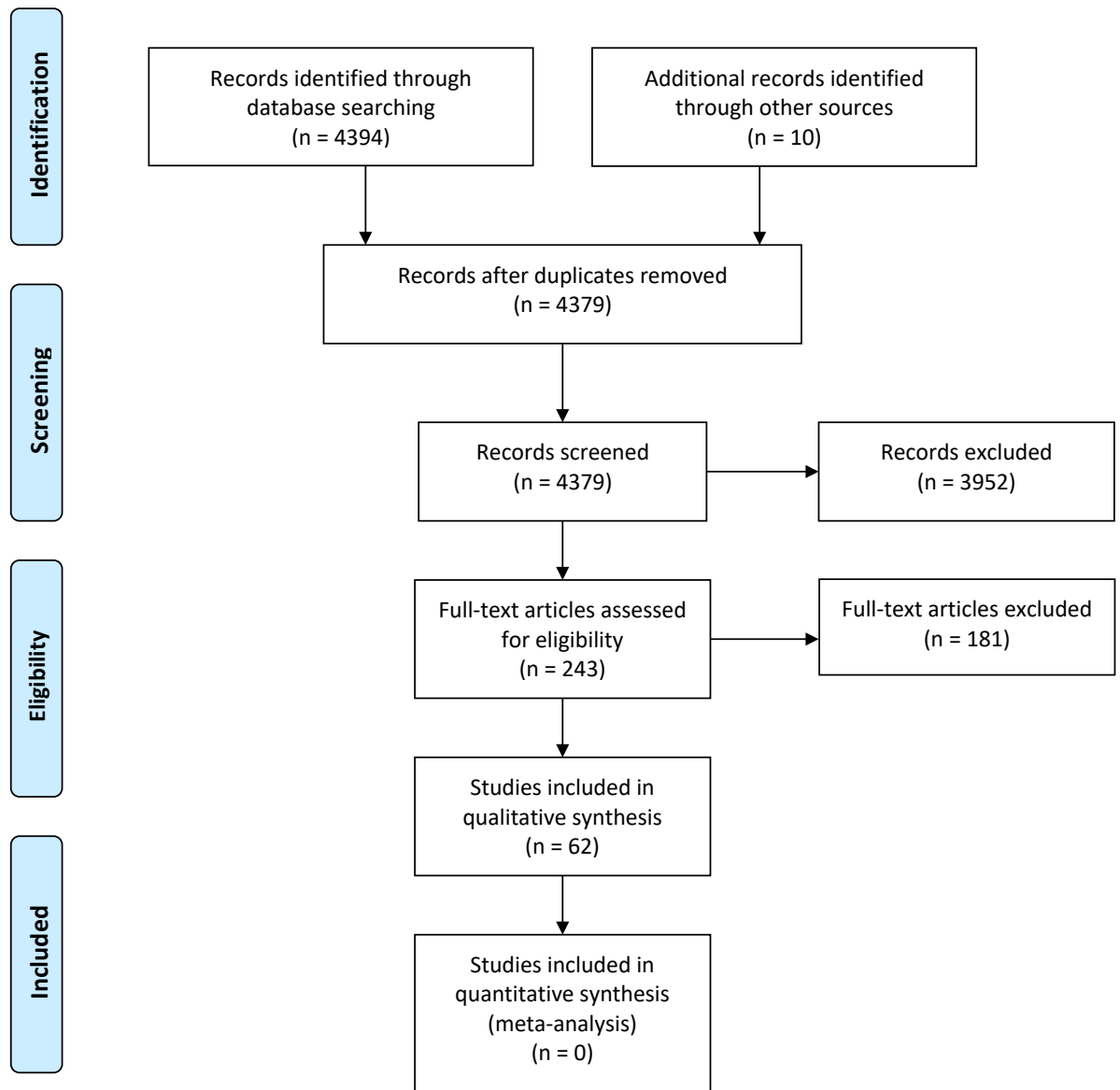


Figure 10 PRISMA flow diagram of study selection.

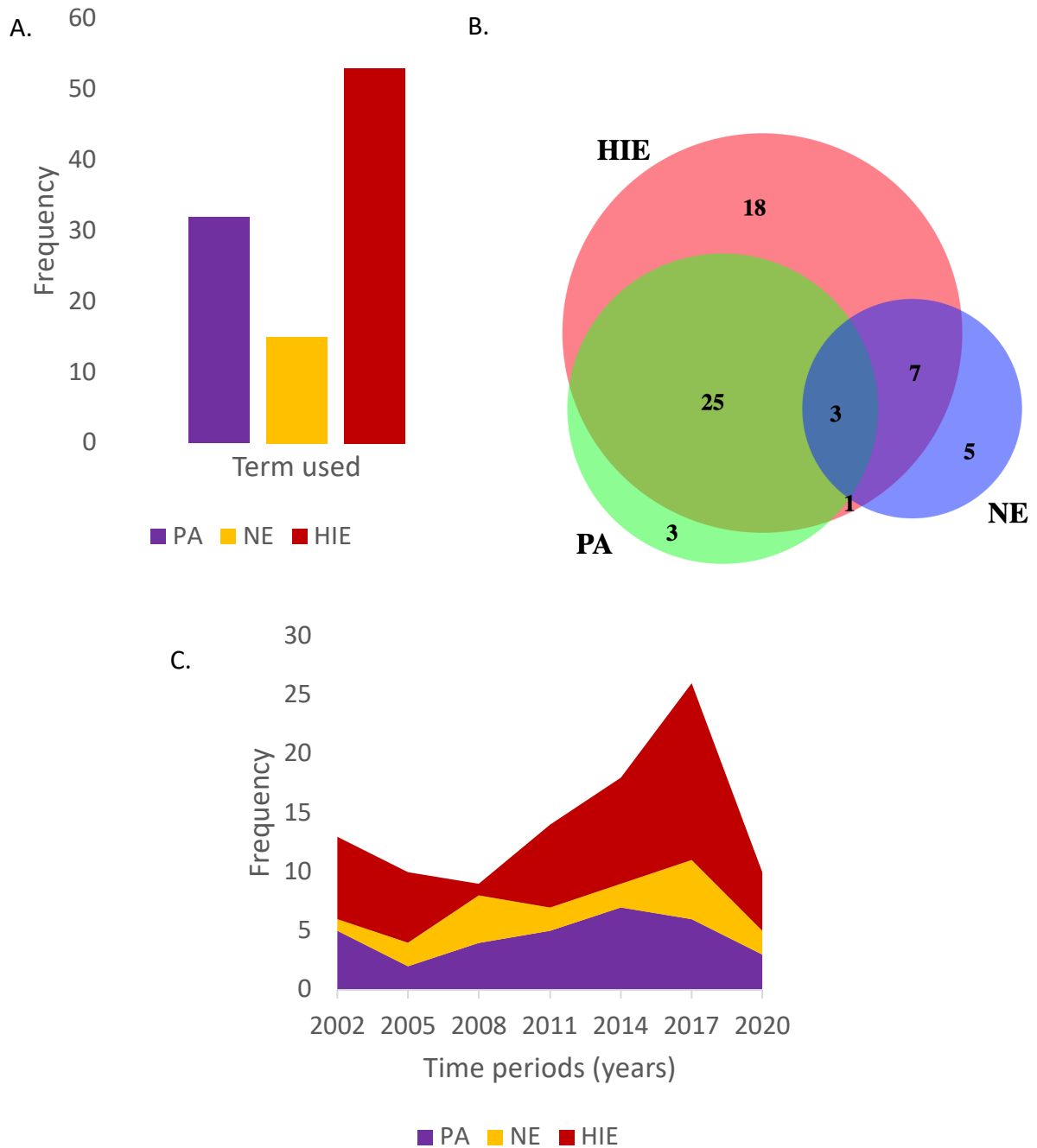
## Terminology

HIE was the most frequently used term to describe the condition, used in 53 of the 62 papers (85%), PA was the next most frequently used term, used in 32 of the 62 papers (52%), and NE was only used in 16 of the 62 papers (26%) (Figure 11A).

Many of the papers used more than one term interchangeably. Most frequently, both HIE and PA were used to refer to the condition in 25 studies (40%), followed by HIE and NE in 7 studies (11%), less frequently by NE and PA in only 1 study (2%), and finally 3 studies (5%) used all the terms interchangeably (Figure 11B).

## Terminology trends over time

Over the past 20 years, while debate regarding the appropriate terminology has persisted, there has not been a significant change in the frequency of terminology used. HIE remained the most frequently used term to describe the condition, used in 50-60% of all studies from 1998-2020 except for a very short sharp decline to 11% from 2005-2008, but recovered immediately in the following period from 2009-2011 to >50% (Figure 11C). PA ranged from a low frequency of use of 20% from 2005-2008 to a high frequency of use of 44% in 2008-2011. NE was initially increasing in frequency of use, rising from 8% (1998-2002) to 20% (2005-2005) and then to 44% (2005-2008) before decreasing again to a fluctuating frequency of between 11-20% from 2008 to 2020.



**Figure 11 Terminology used in randomised controlled trials in neonatal encephalopathy.** The frequency of terminology used in included studies (A), proportional Venn diagram of the frequency and overlap of terms used in included studies, created using BioVenn software (B), and trends in the frequency of terminology used from 1998 – 2020 (C).

## Definitions

We extracted the definition used or referenced in each study. Only 7 studies defined the term used or referenced an existing definition of NE or HIE or PA (Table 1) [54, 140, 176, 204, 227-285], outside of the clinical criteria employed. Therefore, it was not possible to compare the frequency of currently suggested definitions of NE/HIE/PA. Most studies initially discussed the prevalence and incidence of the condition and subsequently defined the condition by the participant eligibility criteria.

Study	NE	HIE	PA	Definition
Ahmad 2018		+	+	WHO 2007: 'the failure to initiate and sustain breathing at birth'
Aker 2019		+		Defined by clinical criteria
Akisu 2003		+	+	Defined by clinical criteria
Akula 2015	+			Defined by clinical criteria
Aly 2009		+		Defined by clinical criteria
Aly 2015		+	+	Defined by clinical criteria
Atici 2015		+		Defined by clinical criteria
Avasiloaiei 2013		+	+	Perinatal asphyxia occurs when an antenatal, intranatal or postpartum neurologic insult or any combination of the three leads to: (i) hypoxemia (decreased oxygen flow to the fetus/ newborn); (ii) hypercapnia (altered O <sub>2</sub> /CO <sub>2</sub> exchange); and (iii) ischemia (inadequate perfusion of tissues and organs)
Azzopardi 2009			+	Defined by clinical criteria
Azzopardi 2013	+	+	+	Defined by clinical criteria
Baserga 2015	+	+		Defined by clinical criteria
Battin 2001		+	+	Defined by clinical criteria
Benders 2006		+	+	Defined by clinical criteria
Bharadwaj 2012		+	+	Defined by clinical criteria
Bhat 2009		+	+	Defined by clinical criteria
Celik 2015		+		Defined by clinical criteria
Das 2017			+	Defined by clinical criteria
Eicher 2005	+	+		Defined by clinical criteria
Farargy 2019		+		Defined by clinical criteria
Filipi 2017		+		Defined by clinical criteria
Gane 2014		+	+	Defined by clinical criteria
Gluckman 2005	+	+		Defined by clinical criteria
Groenendaal 2002		+	+	Defined by clinical criteria
Gunes 2007		+	+	Defined by clinical criteria
Gunn 1998		+	+	Defined by clinical criteria
Horn 2006		+		Hypoxic ischaemic insults during labour [and the] brain injury that occurs in this way is an evolving process and the clinical manifestation of this injury is termed hypoxic ischaemic encephalopathy'
Ichiba 2002		+	+	Defined by clinical criteria
Jacobs 2011		+		Defined by clinical criteria
Joy 2013		+	+	Defined by clinical criteria
Laptook 2017	+	+	+	Defined by clinical criteria
Li 2009		+	+	Defined by clinical criteria
Lin 2006		+	+	Defined by clinical criteria
Lv 2017	+	+		Group of Neonatology, Chinese Pediatric Society, Chinese Medical Association (2005) Diagnostic criteria for neonatal hypoxic-ischemic encephalopathy
Maiwald 2019	+	+	+	Defined by clinical criteria
Malla 2017		+	+	Defined by clinical criteria
Nair 2009			+	Defined by clinical criteria
Nunez-Ramiro 2019		+	+	Defined by clinical criteria
Prakash 2016		+	+	Defined by clinical criteria
Rahman 2015		+		Defined by clinical criteria
Rakesh 2017		+	+	Defined by clinical criteria
Robertson 2008	+	+	+	Defined by clinical criteria
Sami El Shimi 2014		+	+	Defined by clinical criteria
Shankaran 2002	+	+		Defined by clinical criteria
Shankaran 2005		+	+	Defined by clinical criteria
Shankaran 2014		+		Defined by clinical criteria
Shankaran 2017		+		Hypoxic-ischemic encephalopathy represents a subset of neonatal encephalopathy (not defined)', otherwise defined by clinical criteria
Simbruner 2010	+			Defined by clinical criteria
Singh 2004		+		Defined by clinical criteria
Singh 2005		+		Defined by clinical criteria
Srinivasakumar 2015		+		Defined by clinical criteria
Sun 2012		+		Defined by clinical criteria
Tanigasalam 2015		+		Defined by clinical criteria
Tanigasalam 2018	+			Defined by clinical criteria
Thayyil 2013	+			Thompson encephalopathy score >5
Thayyil 2018	+			neonatal encephalopathy – a condition arising from an unexpected lack of cerebral blood flow and oxygen supply to the fetal brain at the time of birth'
Thoresen 2000		+		Defined by clinical criteria
Van Bel 1998		+	+	Defined by clinical criteria
van Rooij 2010		+		Defined by clinical criteria
Velaphi 2013		+	+	Defined by clinical criteria
Wu 2016	+	+		Defined by clinical criteria
Zhou 2010	+	+		Defined by clinical criteria
Zhu 2009		+	+	Defined by clinical criteria

**Table 1 Terminology and definitions reported in included studies. Only 7 studies defined the term used or referenced an existing definition of NE or HIE or PA outside of the clinical criteria employed.**

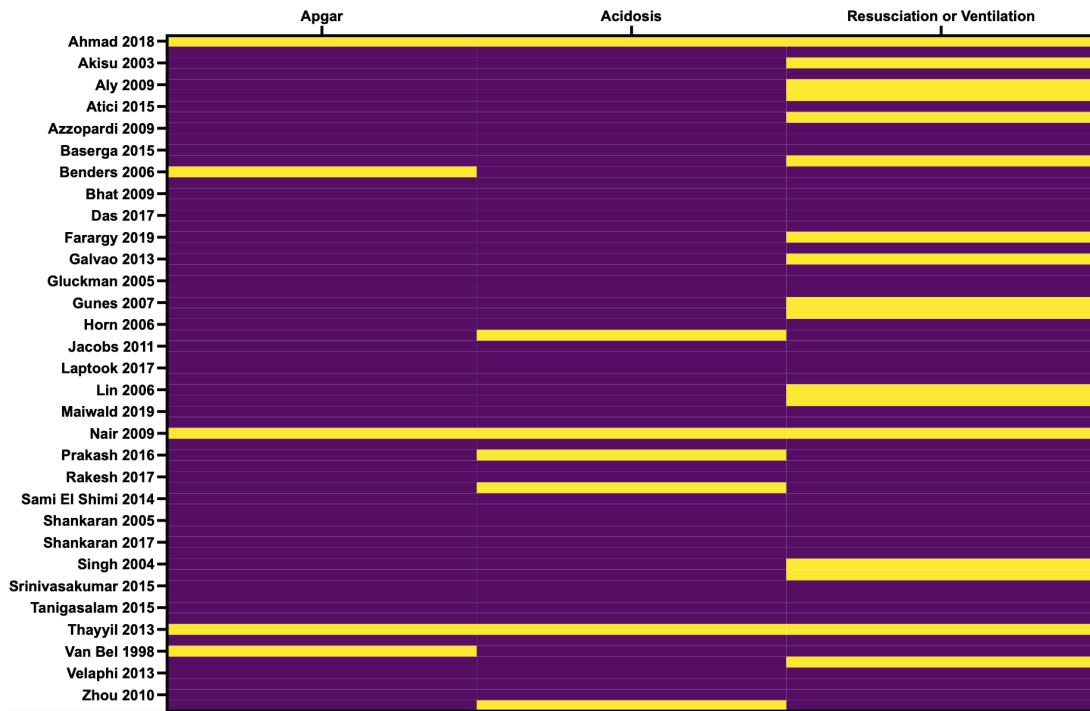
## Inclusion criteria

In trials of interventions for NE, participants frequently had to demonstrate evidence of PA first, and then a neurological assessment followed to examine for evidence of neurological dysfunction. Participants needed to meet the inclusion criteria in both PA and neurological assessment categories to be included in trials, and so we grouped the inclusion criteria into these two categories for this study.

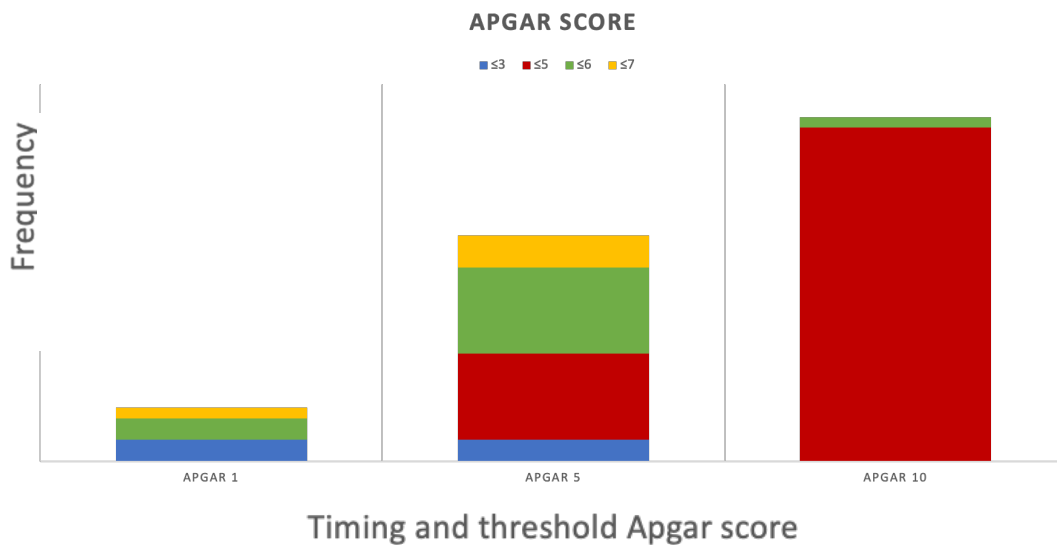
## *Perinatal asphyxia*

PA is defined as the impairment of gas exchange around the time of before, during, or immediately after birth which, if prolonged, can lead to “progressive hypoxaemia, hypercapnia, and significant metabolic acidosis [1]. All included studies required postnatal evidence of perinatal asphyxia which was considered to be a reduced Apgar score in the first few minutes of life, evidence of metabolic acidosis on the umbilical cord blood gas or first postnatal gas, or the need for resuscitation in the period immediately after birth.

Apgar score is an ‘index of health’ [286] applied at various specified intervals in the immediate postnatal period that guides the need for resuscitation [287]. It provides a score out of 10, and the higher the score the better the condition of the neonate and the less likely there is a need for resuscitation. Apgar score was the most frequently used assessment of PA, used in 57 of 62 studies (92%) (Figure 12) [54, 140, 176, 204, 227-252, 254-285].



**Figure 12** Frequency of inclusion criteria used as evidence of perinatal asphyxia for participants in trials for NE. Apgar score was the most frequently used assessment of PA, used in 57 of 62 studies (92%), followed by metabolic acidosis on the umbilical cord blood gas or first postnatal blood gas, used in 51 of 62 studies (82%), and finally need for resuscitation or ventilation was used as a criterion in 45 of 62 studies (73%).



**Figure 13** Frequency of timing and threshold Apgar scores required for inclusion as a participant in trials for NE.

However, the timing and application of the Apgar score varied between studies. Assessment at 10 minutes was the most frequently employed, used in 32 of 57 studies (56%), next was assessment at 5 minutes, used in 21 of 57 studies (37%), and finally assessment at 1 minute was only used in 6 of 57 studies (11%) (Figure 13). Only 2 studies (4%) included assessment at multiple time points. A threshold score  $\leq 5$  was most frequently employed at 10 minutes, used in 32 of 33 studies (97%). However, there was much greater variability in threshold score at 1- and 5- minute assessments. At 5-minute assessment a score  $\leq 5$  was used in 8 of 21 studies (38%), a score  $\leq 6$  in 8 of 21 studies (38%), a score  $\leq 7$  in 3 of 21 studies (14%), and a score  $\leq 3$  in 2 of 21 studies (10%). At 1 minute a threshold score of  $\leq 3$  was used in 3 of 6 studies (50%), a score of  $\leq 6$  in 2 of 6 studies (33%) and  $\leq 7$  in 1 of 6 studies (17%).

The next most frequently employed criterion used as evidence of perinatal asphyxia was metabolic acidosis on the umbilical cord blood gas or first postnatal blood gas, used in 51 of 62 studies (82%) (Figure 12). Both low pH and high base excess (BE) were considered evidence of metabolic acidosis, however low pH was employed in 51 of 55 studies (93%) compared to high BE which was used in 41 of 55 studies (75%). 41 of 55 studies (75%) accepted either criterion as evidence of metabolic acidosis. There was variation in the application of threshold scores.

The most severe pH threshold score,  $\leq 7.0$ , was the most frequently employed in 33 of 51 studies (65%) (Figure 14). A sole threshold pH of  $\leq 7.0$  or a pH between 7.01 – 7.15 was

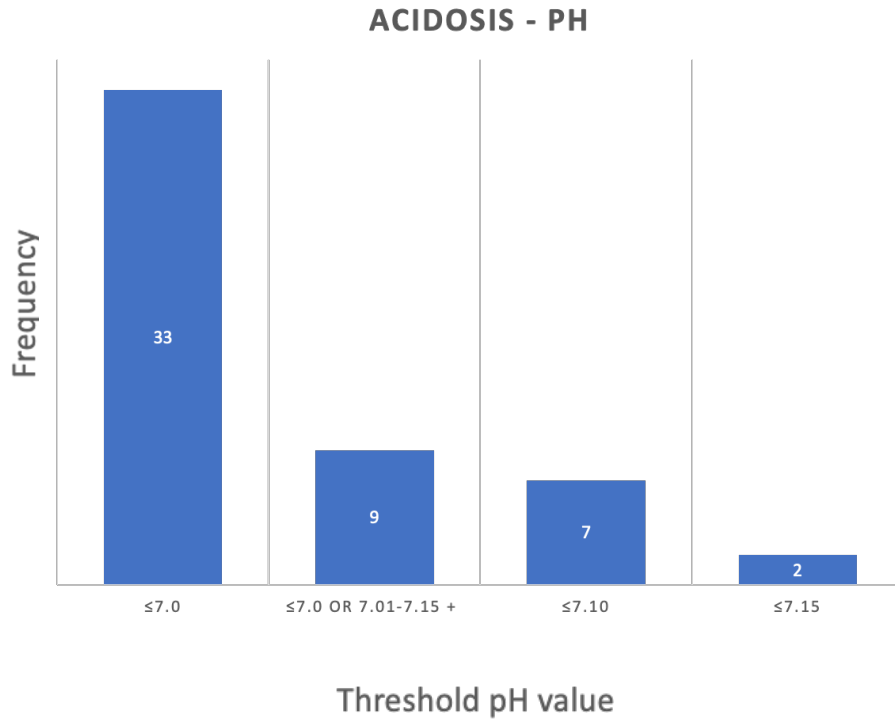


acceptable if further criteria were met in 9 of 51 studies (18%). Participants with a pH  $\leq 7.1$  were eligible for inclusion in 7 of 51 studies (14%), and a pH  $< 7.15$  in 2 of 51 studies (4%).

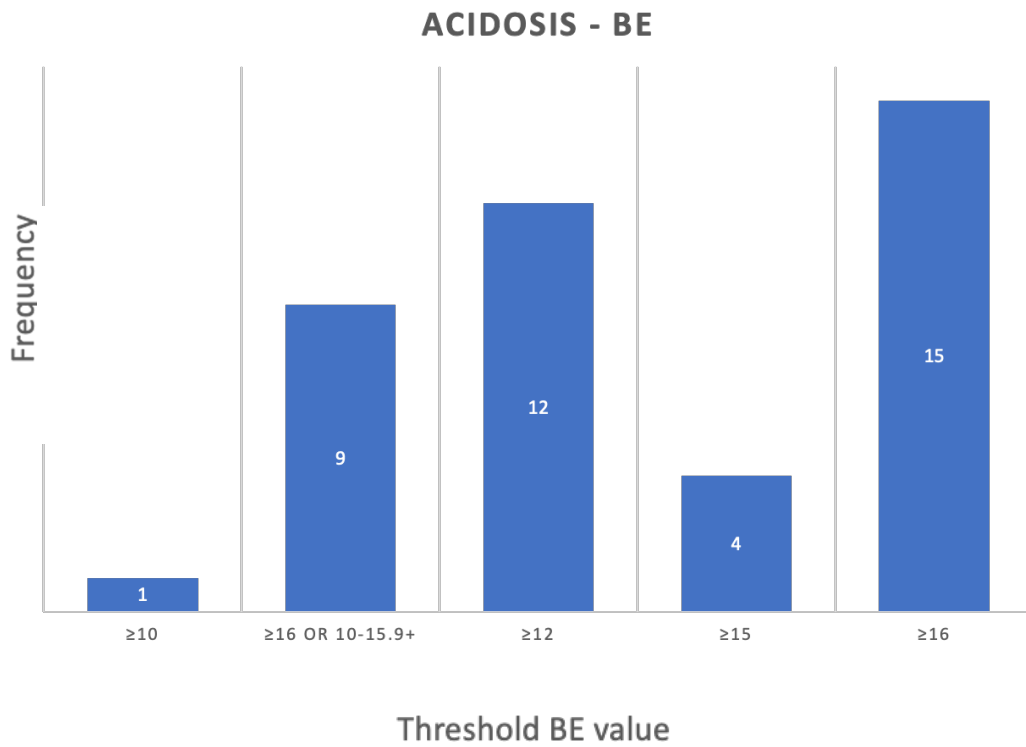
The most severe BE threshold score,  $\geq 16$ , was the most frequently employed in 15 of 41 studies (37%), and a score  $\geq 15$  was used in 4 of 41 studies (10%), a score  $\geq 12$  was used in 12 of 41 studies (29%), and 1 study used a threshold score of  $\geq 10$  (2%) (Figure 15).

Participants were eligible with a sole threshold BE of  $\geq 16$ , or a BE between 10 – 15.9 if further criteria were met in 9 of 41 studies (22%).

A need for resuscitation or ventilation was used as a criterion in 45 of 62 studies (73%) (Figure 12). A need for resuscitation was used in all 45 studies (100%), however the need for ventilation which was used as a criterion in 23 of 45 studies (51%).



**Figure 14** Frequency of threshold pH value required for inclusion as a participant in trials for NE.



**Figure 15** Frequency of threshold BE value required for inclusion as a participant in trials for NE.

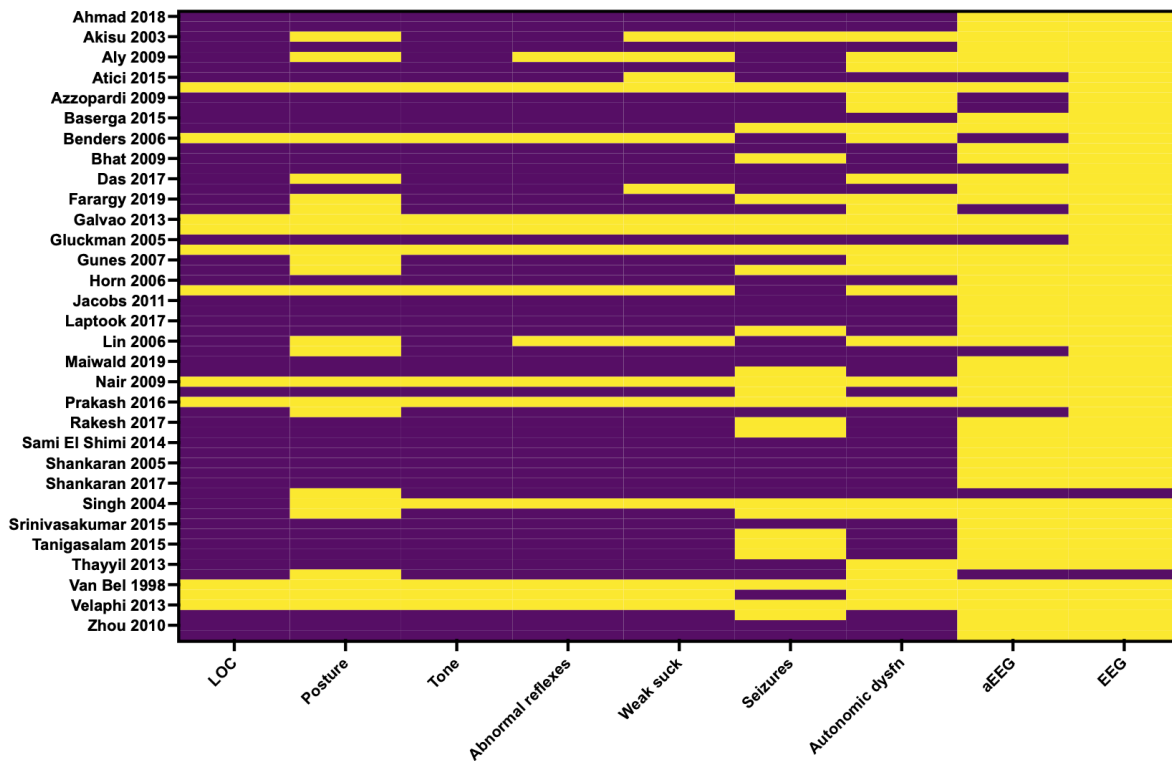
### *Neurological examination*

There are major differences in the application of neurological assessments between RCTs (Table 2) [54, 140, 176, 204, 227-252, 254-285]. Several classifications and scoring systems have been developed for the neurological assessment in the first few minutes and hours of life. The most frequently used include Sarnat staging [288], several modified Sarnat staging, Thompson score [289], and the neurological criteria from the NICHD [268] or TOBY therapeutic hypothermia studies [204]. Very few RCTs explicitly referenced a classification or scoring system as the direct criteria for eligibility for inclusion in a RCT, however many referenced a system and extracted components to form their own eligibility criteria. Furthermore, while some early RCTs based their NA on modifications of existing systems, the neurological assessment from the RCT have later been used in other RCTs in NE/HIE/PA.

The most frequently used criterion from the neurological assessment was a reduced level of consciousness, included in of studies 51 of 62 studies (82%), followed by reduced tone included in 50 studies (81%), abnormal reflexes in 48 studies (77%), and weak suck in 45 studies (73%) (Figure 16) [54, 140, 176, 204, 227-252, 254-285]. Less frequently employed criteria included abnormal posture, used in 37 studies (60%), autonomic dysfunction in 35 studies (56%), and aEEG used in 11 studies (18%).

Study name	Neurological assessment
Ahmad 2018	Thompson score used, no score specified for inclusion or exclusion
Aker 2019	NICHD 2005, Sarnat & Thompson for severity
Akisu 2003	Sarnat for severity - mild excluded
Akula 2015	Coolcap or NICHD criteria, Sarnat for severity
Aly 2009	Sarnat for severity
Aly 2015	Sarnat for severity - mild excluded
Atici 2015	ACOG 2004 criteria, modified Sarnat for severity - mild excluded
Avasiloiabei 2013	ACOG 2004 criteria
Azzopardi 2009	Own criteria (TOBY trial)
Azzopardi 2013	Own criteria, similar to TOBY trial but not referenced, Thompson severity score
Baserga 2015	NICHD 2005 trial criteria
Battin 2001	Own criteria
Benders 2006	Ongoing need for resus beyond 5 minutes, aEEG criteria
Bharadwaj 2012	Sarnat for severity - only moderate - severe included
Bhat 2009	Portman et al and Sarnat, Sarnat for severity
Celik 2015	ACOG criteria, modified Sarnat for severity
Das 2017	Sarnat for severity - mild excluded
Eicher 2005	Sarnat for severity
Farargy 2019	Sarnat for severity - mild excluded
Filipi 2017	CRIB score - mild excluded
Gane 2014	"evidence of encephalopathy", not specified further
Gluckman 2005	Sarnat - moderate or severe only included
Groenendaal 2002	Sarnat for severity, not specified for inclusion or exclusion
Gunes 2007	Sarnat for severity, not specified for inclusion or exclusion
Gunn 1998	"encephalopathy consisting of lethargy/stupor, hypotonia, abnormal reflexes including an absent or weak suck"
Horn 2006	Thompson score $\geq 2$
Ichiba 2002	"failure to initiate spontaneous respiration at 10 min after birth because of asphyxia, or the presence of clinically apparent seizures within 24 h after birth"
Jacobs 2011	Sarnat - moderate or severe only included
Joy 2013	Sarnat - moderate or severe only included
Laptook 2017	NICHD 2005 trial criteria
Li 2009	Sarnat - moderate or severe only included
Lin 2006	"clinical signs of postpartum encephalopathy (decreased muscle tone, lethargy, coma, or seizures) starting within 6 h after birth"
Lv 2017	"diagnostic criteria of neonatal HIE formulated by the Chinese Medical Association"
Maiwald 2019	"must meet two out of the following four criteria for potentially evolving encephalopathy to participate in the study: 1) altered state of consciousness (reduced or absent response to stimulation or hyperexcitability); 2) severe muscular hypotonia or hypertonia; 3) absent or insufficient spontaneous respiration (i.e. gasping only) with need for respiratory support at 10 min postnatally and/or 4) abnormal primitive reflexes (absent suck/gag/ corneal/Moro reflex) or abnormal movements (i.e. potential clinical correlates of seizure activity)"
Malla 2017	Modified Sarnat, moderate or severe only, required $\geq 3/6$ criteria
Nair 2009	"clinical evidence of encephalopathy observed in the first 7 days of postnatal life" not specified further
Nunez-Ramiro 2019	Modified Sarnat
Prakash 2016	Modified Sarnat
Rahman 2015	Reduced LOC + $\geq 1$ of hypotonia, abnormal reflexes or absent or weak suck. Modified Sarnat - moderate or severe only.
Rakesh 2017	Modified Sarnat
Robertson 2008	Thompson score $>5$
Sami El Shimi 2014	Thompson score, mild excluded
Shankaran 2002	Own, NICHD classification
Shankaran 2005	Own, NICHD classification
Shankaran 2014	NICHD 2005 trial criteria
Shankaran 2017	NICHD 2005 trial criteria
Simbruner 2010	Own criteria from neo.nEURO.network
Singh 2004	"overt neurological syndrome in form of alteration of tone and/or sensorium within the first six hours of life" (Sarnat staging)
Singh 2005	"features of encephalopathy in the form of alterations of tone, deep tendon reflexes, primitive reflexes and sensorium" (Sarnat staging)
Srinivasakumar 2015	NICHD criteria or seizures
Sun 2012	Sarnat (mild - severe)
Tanigasalam 2015	Sarnat criteria (mild - severe)
Tanigasalam 2018	Sarnat - moderate or severe only included
Thayyil 2013	Thompson score $>5$
Thoresen 2000	"Signs of encephalopathy, eg, lethargy, stupor, hypotonia, absent suck, and clinical seizures"
Van Bel 1998	Need for resuscitation for $>2$ minutes, Sarnat for severity
van Rooij 2010	Sarnat for severity - mild excluded
Velaphi 2013	Sarnat for severity
Wu 2016	Sarnat for severity - moderate or severe only, mild excluded, req $\geq 3/6$ sarnat criteria
Zhou 2010	Sarnat (mild - severe)
Zhu 2009	Sarnat - moderate or severe only included

**Table 2 Summary of the neurological assessment in each RCT. There is very wide variation in application of neurological exam criteria but the most frequently employed were Sarnat staging, several modified Sarnat staging, Thompson score, and the neurological criteria from the NICHD or TOBY therapeutic hypothermia studies.**



**Figure 16** Frequency of inclusion criteria used as evidence of neurological dysfunction for participants in trials for NE. The most frequently used criterion was reduced level of consciousness (82%), followed by reduced tone (81%), abnormal reflexes (77%), and weak suck (73%). Less frequently employed criteria included abnormal posture (60%), autonomic dysfunction (56%), and aEEG (18%).

## Exclusion criteria

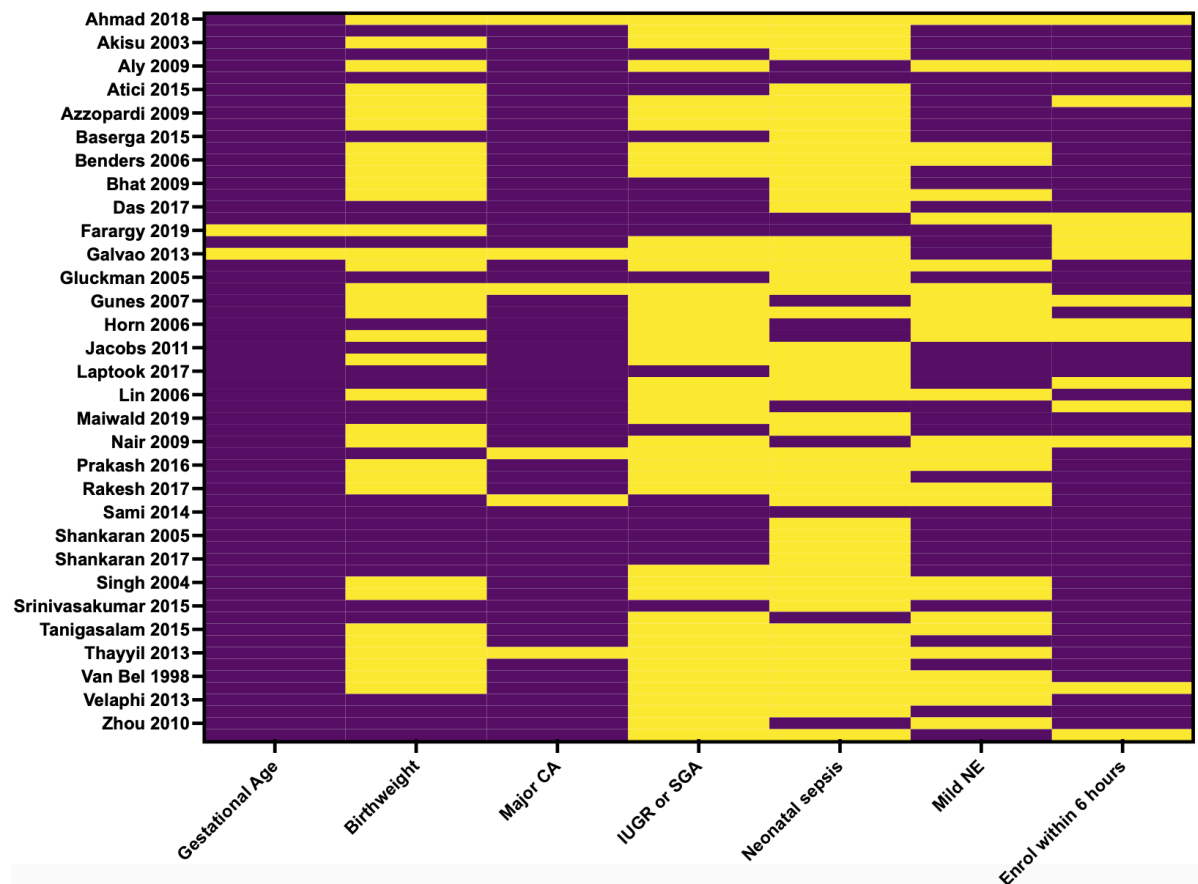
Gestational age was the most frequently employed exclusion criteria, used in 60 of 62 studies (97%), followed by the presence of major congenital abnormalities, used in 56 of 62 studies (90%), and the need for identification and diagnosis within 6 hours of birth, used in 47 of 62 studies (76%) (Figure 17) [54, 140, 176, 204, 227-252, 254-285]. Participants with mild NE were excluded in 35 of 62 studies (56%). Participants with low birthweight were excluded in 27 of 62 studies (44%) and participants born small for gestational age were excluded in 19 of 62 studies (31%). Participants with neonatal sepsis were excluded in 12 of 62 studies (19%).

Term gestation of  $\geq 37$  weeks gestational age at birth was the most frequency employed threshold for participation in RCT, used in 27 of 60 studies (45%), followed by late preterm gestational ages of  $\geq 36$  weeks GA at birth, used in 21 of 60 studies (35%), and  $\geq 35$  weeks GA at birth, used in 7 of 60 studies (12%) (Figure 18). The further extreme GA thresholds of  $\geq 34$  and  $\geq 38$  weeks at birth were used much less frequently, in 4 of 60 (7%) and 1 of 60 studies (2%) respectively.

The most frequently employed minimum birthweight threshold employed was  $\geq 1800$ grams, used in 13 of 27 studies (48%), followed by a minimum threshold of  $\geq 2500$ grams, used in 7 of 27 studies (26%), and finally a threshold of  $\geq 2000$ grams was used in 6 of 27 studies (22%) (Figure 19).

## Other eligibility criteria

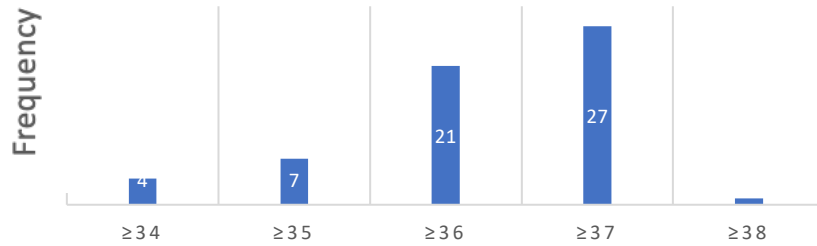
Several studies employed additional eligibility criteria outside of postnatal evidence of perinatal asphyxia and neurological dysfunction on clinical examination. The most frequently used was evidence of a perinatal sentinel event (PSE), an acute event in the perinatal history that possibly compromises placental blood flow [290], which was used in 29 of 62 studies (47%) (Figure 20). Electrophysiological monitoring by electroencephalography (EEG) or amplitude-integrated EEG (aEEG) to detect abnormal cerebral function was used in 2 of 62 (3%) and 11 of 62 studies (18%) respectively. Evidence of multi-organ dysfunction was employed as a criterion in 8 of 62 studies (13%).



**Figure 17** Frequency of exclusion criteria used for participants in trials for NE. Gestational age was the most frequently employed exclusion criteria (97%), followed by the presence of major congenital abnormalities (90%), and the need for identification and diagnosis within 6 hours of birth (76%).



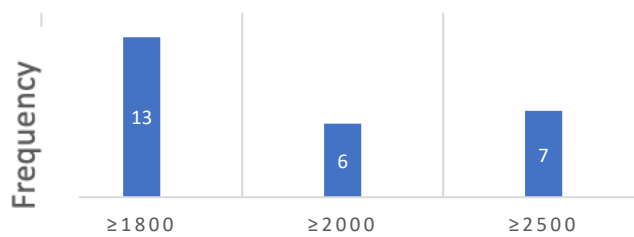
## GESTATIONAL AGE



Threshold gestational age

*Figure 18 Frequency of threshold gestational age in weeks required for exclusion as a participant in trials for NE.*

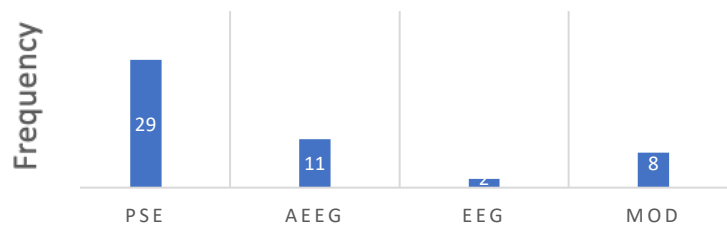
## BIRTHWEIGHT



Threshold birthweight

*Figure 19 Frequency of threshold birthweight in grams required for exclusion as a participant in trials for NE.*

## ADDITIONAL ELIGIBILITY CRITERIA



Additional exclusion criteria used

*Figure 20 Frequency of additional exclusion criteria used for participants in trials for NE.*

## Large trials TH comparison

As the only intervention widely available for patients with NE, we selected the large trials of TH with >100 participants for comparison of their eligibility criteria and control group mortality (Table 3) [204, 246, 253, 268]. These trials provide the eligibility criteria for TH in many national guidelines in patients with NE [219, 220]. Studies were in universal agreement regarding the Apgar score threshold of  $\leq 5$  at 10 minutes of life. Three studies required evidence of metabolic acidosis on cord blood gas or first postnatal blood gas of a pH value of  $\leq 7.0$ , and one study accepted a pH of 7.01-7.15 if further criteria were met. Two studies required a BE of  $\geq 16$ , 1 a BE of  $\geq 12$ , and one accepted a BE of 10-15.9 if further criteria were met. Studies were in universal agreement regarding the use of the need for resuscitation at 10 minutes of life as an inclusion criterion, however 2 of the studies specified the need for ventilation as part of the resuscitation at 10 minutes of life. Only one study included perinatal sentinel event as an inclusion criterion. All studies were in universal agreement regarding the use of modified Sarnat staging (although not explicitly stated in either the TOBY or NICHD studies) as the NA for inclusion in trials for NE. The application of modified Sarnat staging differed significantly between studies, with any staging accepted in one study, only those with moderate-severe encephalopathy accepted in another, reduced level of consciousness and one further sign required in one study, and signs in 3 categories required in the final study. Two studies included abnormalities on aEEG as a criterion for inclusion. Studies were similar in the threshold for exclusion based on gestational age at birth, 3 using  $\geq 36$  weeks and 1 using  $\geq 35$  weeks, and in the threshold for exclusion based on birthweight, 2 using  $\leq 1800$  grams and one using  $\leq 2000$  grams. All studies excluded patients

with major congenital abnormalities. All studies excluded patients not enrolled within 6 hours of birth. Despite the many similarities in eligibility criteria across these studies, the control group mortality varied by 12% between studies. Given the similarities in clinical management between studies, differences in the eligibility criteria may be a significant contributing factor to this variation in control group mortality.

#### Control group mortality

Control group mortality varied significantly between included studies, from the lowest rate of 2% to the highest rate of 67% (Table 4) [54, 140, 176, 204, 227-252, 254-285]. The control group was treated with TH in 16 of 60 studies (27%). The control group mortality in those treated with TH was substantially different compared to the entire cohort, with the lowest mortality rate of 0% to the highest mortality rate of 33%. The duration of follow up, varied substantially from a follow up duration of 3 days to 24 months.

Criteria	Coolcap	NICHD	TOBY	ICE
Apgar		10 ≤5	10 ≤5	10 ≤5
Acidosis	ph ≤7.0 or BE ≥16	pH ≤7.0/7.01-7.15+ or ≥16/10-15.9+		ph ≤7.0 or BE ≥16
Resus	≥10 minutes	Ventilation ≥10 minutes	≥10 minutes	Ventilation ≥10 minutes
PSE	No	Yes	No	No
Neurological	Sarnat -mod/severe only	1+ signs in ≥3/6 categories	Altered LoC + 1 other feature	Sarnat - any grade
aEEG	Abnormal background or seizures	No	Abnormal background or seizures	No
GA	≥36	≥36	>36	≥35
Birthweight	<1800	<1800	N/A	<2000
MCA	Yes	Yes	Yes	Yes
Enrol by 6 hours	Yes	Yes	Yes	Yes
Mortality (%)		38	27	27

**Table 3 Eligibility criteria and control group mortality in large trials of therapeutic hypothermia for neonatal encephalopathy.** PSE – perinatal sentinel event; aEEG - Amplitude-integrated electroencephalography; GA- gestational age; MCA – major congenital abnormality; BE – base excess.

Study name	Year	n=	Intervention	Control	TH	Control Mortality n (%)	Duration of follow up
Ahmad	2018	80		Melatonin	No	14 (35)	Death prior to hospital discharge
Aker	2019	50		TH	No	1 (4)	Death prior to hospital discharge
Akisu	2003	21		TH (SHC)	No	2 (20)	Death within the first 3 days of life
Akula	2015	100		TH (Device in transport)	Yes	6 (12)	Death prior to hospital discharge
Aly	2009	60		Ascorbic acid and ibuprofen	No	10 (33)	Not specified
Aly	2015	30		Melatonin	Yes	4 (27)	Death within the first 6 months of life
Atici	2015	30		SHC vs WBC	Yes	1 (8)	Death prior to hospital discharge
Avasiloaiei	2013	67		Phenobarbital or EPO	No	4 (17)	Not specified - participants followed up to 18 months
Azzopardi	2009	325		TH	No	44 (27)	Not specified - participants followed up to 18 months
Azzopardi	2016	92		Xenon	Yes	9 (20)	Death prior to hospital discharge
Baserga	2015	30		Darbepoetin	Yes	1 (10)	Death within the first 7 days of life
Battin	2001	40		SHC	No	3 (15)	Death within the first 7 days of life
Benders	2006	32		Allopurinol	No	10 (67)	Death prior to hospital discharge
Bharadwaj	2012	124		TH (Gel packs)	No	6 (10)	Death prior to hospital discharge
Bhat	2009	40		MgSO4	No	2 (10)	Death prior to hospital discharge
Celik	2015	31		SHC vs WBC	N/A	4 (33)	Death within the first 12 months of life
Das	2017	60		TH	No	9 (30)	Death within the first 30 months of life
Eicher	2005	65		TH	No	14 (42)	Death within the first 12 months of life
Farargy	2019	60		MgSO4 & melatonin vs melatonin alone	Yes	N/A	Neither group considered to have standard therapy
Filipi	2017	44		Topiramate	Yes	2 (9)	Death within the first 24 months of life
Gane	2014	122		TH (Gel packs)	No	8 (16)	Death within the first 12 months of life
Gluckman	2005	234		SHC	No	42 (38)	Death within the first 18 months of life
Groenendaal	2002	22		MgSO4	No	6 (43)	Death within the first 24 months of life
Gunes	2007	60		Allopurinol	No	3 (11)	Death within the first 12 months of life
Gunn	1998	22		SHC	No	2 (20)	Death within the first 18 months of life
Horn	2006	20		SHC (solid ice cap)	No	Not reported	N/A
Ichiba	2002	34		MgSO4	No	1 (6)	Death within the first 14 days of life
Jacobs	2011	221		TH	No	42 (38)	Death within the first 24 months of life
Joy	2013	116		TH	No	4 (7)	Death prior to hospital discharge
Laptook	2017	168		TH (beyond 6 hours of life)	No	5 (6)	Death within the first 22 months of life
Li	2009	93		TH (initiated within the first 10 hours)	No	3 (7)	Death within the first 18 months of life
Lin	2006	62		TH (SHC)	No	2 (7)	Death within the first 10 days of life
Lv 2017	2017	42		EPO	Yes	0 (0)	Death within the first 9 months of life
Maiwald	2019	N/A		Allopurinol	Yes	N/A	Protocol only
Malla	2017	100		EPO	No	8 (16)	Death within the first 19 months of life
Nair	2009	108		Pyritinol	No	1 (2)	Death within the first 12 months of life
Nunez-Ramiro	2019	180		Topiramate	Yes	10 (19)	Death prior to hospital discharge
Prakash	2016	120		MgSO4	No	Not reported	Only a composite of death or disability reported
Rahman	2015	60		MgSO4	Yes	5 (16)	Death prior to hospital discharge
Rakesh	2017	120		TH	No	16 (28)	Death within the first 6 months of life
Robertson	2008	36		TH	No	1 (7)	Death prior to hospital discharge
Sami El Shimi	2014	30		EPO	Yes	2 (20)	Death within the first 3 months of life
Shankaran	2002	19		TH	No	3 (30)	Death prior to hospital discharge
Shankaran	2005	239		TH	No	29 (27)	Death within the first 22 months of life
Shankaran	2014	364		TH (2x2 comparison of longer and deeper)	Yes	7 (7)	Death within the first 22 months of life
Simbruner	2010	129		TH	No	33 (57)	Death within the first 21 months of life
Singh	2004	45		Phenobarbital	No	3 (15)	Death prior to hospital discharge
Srinivasakumar	2015	72		Treatment of electrographic vs clincial seizures only	Yes (some)	2 (6)	Death within the first 24 months of life
Sun	2012	56		SHC	No	1 (4)	Death prior to hospital discharge
Tanigasalam	2015	120		TH	No	30 (50)	Death prior to hospital discharge
Tanigasalam	2018	80		Fluid restriction	Yes	3 (8)	Death within the first 6 months of life
Thayyil	2013	33		TH	No	2 (13)	Death prior to hospital discharge
Thayyil	2018	N/A		TH	No	N/A	Protocol only
Thoresen	2000	9		TH (intervention study but not a RCT)	No	Not reported	N/A
Van Bel	1998	22		Allopurinol	No	6 (55)	Death prior to hospital discharge
van Rooij	2010	42		Treatment of aEEG seizures	No	7 (50)	Death in the neonatal period
Velaphi	2013	94		Phenobarbital	No	7 (16)	Death prior to hospital discharge
Wu	2016	50		EPO	Yes	5 (19)	Death prior to hospital discharge
Zhou	2010	256		TH (SHC)	No	27 (29)	Death within the first 18 months of life
Zhu	2009	167		EPO	No	4 (5)	Death within the first 18 months of life

**Table 4 Intervention, follow up duration, and control group mortality in interventional trials.**

## Discussion

NE is a condition with a complex pathophysiology, a wide spectrum of severity, and with only one specific well-evidenced intervention which requires very early identification for treatment to be initiated, often before many diagnostic test results are available. It is also much more prevalent in resource-limited settings where there is limited access to diagnostic tests and where the intervention, TH, is neither safe nor feasible [5, 291]. All of these features mean it is very difficult to agree a universal definition and the criteria for diagnosis.

Despite persistent recommendations from various organisations suggesting use of the term NE, including the largest effort to date [1], and HIE to be reserved for a cause-specific subgroup of those with NE, HIE remains the most prevalent term used to describe the condition. There is no evidence that the ACOG/AAP Taskforce on Neonatal Encephalopathy and Neurologic Outcome [1] recommendation of the definition of and use of the term NE was generally accepted, and the trend is increasingly to use the term HIE. Since the ACOG and the AAP Taskforce on NE have recently defined NE and HIE specifically, it may become more challenging to identify when authors intend to use NE rather than HIE, and when HIE by the Taskforce's definition is being used deliberately. This has implications for understanding the epidemiology of the condition which, to date, has described separate entities with the incidence of NE estimated at 3.0 per 1000 births, and the incidence of HIE 1.5 per 1000 births [292]. There is not universal agreement over the definitions provided by the Taskforce and some criticisms suggest that it is an overly restrictive definition that

attempts to limit the diagnosis for medico-legal purposes. Another criticism is that the definition group is primarily composed of clinicians and does not reflect the input and thoughts of patients, parents, carers, families, and researchers in this field. Therefore, it is critical that there is a universal understanding of what is meant by terms NE or HIE or PA, and that use of the terms is commonly understood. A universal understanding and definition of the meaning of NE would provide diagnostic clarity, improved estimation of incidence, and improved identification of risk factors. This would also improve the clarity on the eligibility criteria for inclusion future trials of interventions for NE.

We found significant variation in control group mortality between studies, varying between 2% and 67% between all studies, and between 27% and 39% even between the largest trials of TH with >100 participants included. Further, since the broad introduction of TH as routine treatment in high-resource settings, there has been an extension of this intervention to patients that were not represented in the trials of TH, such as patients with mild NE [293]. There is increasing concern and evidence of harm from treating patients with TH outside of the originally intended cohorts, such as increased apoptosis in cooled uninjured brains [223]. Further, when the eligibility criteria of RCTs does not represent the subsequent real-world treatment group, and the real-world treatment group differs from the RCT population, then factors outside of the intervention itself may influence the measured effect of the intervention, called heterogeneity of treatment effect (HTE). This limits the generalizability of results [294]. Therefore, it is even more difficult to generalise results from RCTs when the population differs across different studies, even when

differences in eligibility criteria are subtle and the intervention is the same. As the eligibility for the intervention, TH, and more generally for the diagnosis of NE is based on the eligibility criteria from RCTs, this issue is particularly significant for patients with NE.

The Apgar score was developed in 1953 as a method of classifying or grading the overall condition of newborns at birth and it is currently widely used in clinical practice [287]. It comprises a clinical assessment of five components, colour, heart rate, reflexes, muscle tone, and breathing. It provides a score out of ten, with a lower score indicating worse clinical condition at birth. Although the original intention of the Apgar score was as a means of comparison for obstetric practices, types of maternal pain relief, and the effects of resuscitation, in recent years it's use has been extended as a prognostic biomarker for newborns with NE/HIE. Some advantages of the Apgar score includes it's immediate availability, ease of use, and it does not require significant equipment for measurement. This may be one reason why it is the most consistently used inclusion criteria for trials in NE, used 92% of all studies. A threshold of a score  $\leq 5$  at 10 minutes was most frequently employed, however there was substantial variation in the application of timing of assessment and threshold values for inclusion. There is variable evidence of the discriminatory value of the Apgar score. A secondary analysis of the Eunice Kennedy Schreiber trial of TH for NE, which employed a score of  $< 5$  at 10 minutes as an inclusion criteria, found that 30% of participants with a score  $\geq 5$  had died or survived with disability, compared to 65% of those with Apgar score  $< 5$  [295]. A large population-based cohort study demonstrated that among term patients with anoxia a 5 minute Apgar score of 4-6 was



associated with a mortality rate of 54/1000 compared to 0.42/1000 for those with an Apgar score of 7-10 [286]. Another large population-based cohort study demonstrated an association of Apgar score with later diagnosis of cerebral palsy in both 5 and 10 minute Apgar score, but the association was stronger at 10 minutes [296]. ACOG and AAP caution however that Apgar score cannot be used as a consequence of or evidence of asphyxia and is not a predictive of individual risk of mortality or neurologic outcome and that it's role is to give an immediate assessment of the infants status and to assess the response to resuscitation efforts.

Selecting only the most severe cases on evidence of perinatal asphyxia, may fail to provide TH to patients who may benefit from the treatment, and the evidence regarding the use of TH for those with mild NE remains uncertain [224]. VON Ireland 2019 demonstrated that 37.5% of patients that received TH did not have acidosis on either cord nor initial blood gases [297]. Therefore, the population receiving TH at present do not reflect the RCT trial eligibility criteria, and the RCT results may not be generalised to the population outside of the eligibility criteria. Furthermore, if the intention is to identify patients at risk of adverse outcome, only those eligibility criteria that are associated with increased risk of adverse outcome should be included. Threshold values of pH, BE, and Apgar score should reflect current evidence of what values are associated with increased risk of adverse outcome and not only expert consensus or clinical trial eligibility criteria. We found that while evidence of acidosis was a frequently employed eligibility criteria there was wide variation in the application of pH threshold values, and even wider variation in BE threshold values, with the

most severe of each being the most frequently employed. A large systematic review, prior to the introduction of routine treatment with TH, demonstrated strong temporal association between low cord pH and increased neonatal mortality, HIE, and cerebral palsy [298]. More recent evidence suggests that an umbilical arterial cord blood gas pH of  $<7.10$  has a sensitivity of 74% and a specificity of 99%. However, if a threshold value of  $<7.0$  had been employed, 34 of the 69 patients treated with TH would not have been eligible [299]. The study estimates that 25 neonates with a pH 7.0-7.1 would require screening to identify one neonate with moderate-severe encephalopathy and result in a 15% increase in appropriate selection for TH compared to a threshold value of  $<7.0$ . Other recent studies have demonstrated a dose-dependent relationship between base excess (BE) and death or cerebral palsy, using an initial threshold value of  $>12$  [300]. The strength of association with BE  $<12$  is unclear.

We found significant variation in criteria employed for the assessment of neurological dysfunction and very limited consistency in the use of standardised systems. Sarnat staging and Thompson scores, and variations of these classification systems, were among the most frequently employed, however they were both developed for the purpose of prediction rather than classification of severity [301]. Another frequently employed scoring system, NICHD, was however developed for the purpose of classification of severity. Further difficulties arise in the implementation of these criteria. There is significant subjectivity regarding the assessment of many aspects of neurological dysfunction. Among the most frequently employed neurological criteria were reduced level of consciousness,

reduced tone, and abnormal reflexes which were employed in over 75% of included studies. A recent study demonstrated that although there was high levels of inter-observer agreement on the neurological status of a newborn when standardised NE exam criteria were applied, differences in the application of these tests resulted in significant differences in the proportion of newborns eligible for TH [205]. These two issues were identified by the Society for Pediatric Research who recommended standardisation of the neurological exam, training and validation of each examiner, and consistent timing of serial neurological exams for future neuroprotective trials in NE [225]. Furthermore, if there is to be a universal definition of NE and eligibility criteria for future trials, this will have to account for differences in resources in the diagnosis of NE and therefore evidence of metabolic acidosis may not always be available.

Gestational age (GA) was an exclusion criterion that was almost universally applied (97%). Threshold values of GA ranged from >36 or >37 weeks, accounting for 80% of all included studies, and only 20% used a threshold GA of >34 or >35 weeks. There are concerns that TH in preterm patients <35 weeks may result in hypotension, increased oxygen consumption, and respiratory compromise as a result of reduced surfactant production [302]. Despite these concerns early evidence suggests that overall complication rates from TH were similar among those born at 34-35 weeks GA compared to those born at >36 weeks GA, although hypoglycaemia and rewarming prior to the completion of TH were both higher in the earlier preterm group [303]. Mortality rates and white matter injury on MRI were also higher in the preterm group compared to the those born >36 weeks GA. This

was a retrospective review, and we could not identify any trials to date that specifically examined the effects of TH on newborns born at 34-35 weeks GA. Low birthweight (LBW) was a much less frequently employed exclusion criterion, used in just 44% of included studies. Threshold values ranged from <2500gms to <1800gms. There are concerns for TH in LBW patients as hypothermia has been associated with increased risk of IVH and an increased risk of death in the VLBW population [304]. Reassuringly, recent evidence suggests that BW for GA does not appear to influence complication rates or outcomes in TH [305]. RCTs of selective head cooling, employing a threshold BW of <1800gms have also demonstrated a significant positive correlation between lower birth weight and positive better outcomes [306].

Comparison of the eligibility criteria employed in the large trials of TH demonstrated consistency across many criteria however control group mortality still varied significantly. As the study by Walsh et al. demonstrates, small variations in application of eligibility criteria have major consequences and may partially explain this significant variation in control group mortality [205]. For the entire cohort there are too many variables and too wide variation in control group mortality to make any definitive conclusions. It was beyond the scope of this study but a measure of the association between individual eligibility criteria and the control group mortality would provide an indication of the strength of association between the criterion and adverse outcome, and therefore a measure for its justification for inclusion as an eligibility criterion in future trials.

## Chapter 3: Optimal MRI technique to predict long-term prognosis in NE: a systematic review and meta-analysis

### Abstract

**Background:** Neonatal encephalopathy (NE) remains the primary cause long term neurodevelopmental disability in term newborns. An increasing number of interventions are available for newborns at risk of cerebral palsy and other neurodevelopmental conditions. It is critical that high risk patients are recognized early so that interventions can be implemented as early as possible. MRI offers valuable prognostic information in NE, but the predictive value varies by MRI technique. Our objective was to examine the evidence currently available to determine the optimum technique and of MRI in NE.

**Methods:** This meta-analysis was designed according to the Preferred Reporting Items for Systematic Reviews and Meta- Analyses (PRISMA) guidelines and registered with Prospero (CRD42021286461). PubMed, Embase, Web of Science, and the Cochrane database were searched with relevant search terms from January 1966 to July 2020. Observational and interventional studies that included patients over 35 weeks gestational age at birth with any clinical diagnosis of NE, that associated MRI results with neurodevelopmental outcomes, were included. The outcome was a composite of mortality or adverse neurodevelopmental outcome over 12 months of age. Screening, quality assessment and data extraction were completed using Covidence software. Risk of bias assessment was carried out using QUIPS-2 tool. Diagnostic odds ratio (DOR) was calculated using a random-effects model and Meta-analyst software.

**Results:** The literature search provided 3626 results. Following title, abstract and full text screening 114 studies were included in the qualitative assessment, and 59 were included in the meta-analysis. The overall DOR of MRI in the diagnosis of adverse outcome was 23.7 (95% CI 16.7 – 33.5). 49 studies were included in the subgroup of conventional T1/T2 imaging which provided a DOR of 15.4 (95% CI 11.4 to 20.9). 23 studies were included in the diffusion weighted imaging subgroup which provided a DOR of 20.4 (95% CI 10.8 to 38.4). 17 studies were included in the magnetic resonance spectroscopy subgroup which provided a DOR of 45.8 (95% CI 22.7 – 92.4).

**Discussion:** MRI is an excellent prognostic biomarker for outcome in NE. MRS appears to provide the highest DOR but the least consistent results between studies. Further work to examine the causes of heterogeneity and inconsistency between results is required. The optimum timing of MRI and the optimum MRS metabolites also requires examination.

## Introduction

Neonatal encephalopathy (NE) remains a difficult condition to define however it is caused by peripartum factors that ultimately lead to hypoxia and ischaemia (HI) and is identified by disturbed neurological function in the first few hours and days of life. The incidence of NE is difficult to estimate and varies between high and low-middle income countries, but the best estimate is 2-6/1000 births [12]. NE leads to multi-organ dysfunction, but the brain is primarily affected [216]. It is associated with high incidence of mortality (25%) and moderate-severe neurodevelopmental disability (NDD) in survivors (20%) [11], despite routine use of TH in high-income countries [4] for those with moderate or severe encephalopathy. Although the effectiveness of many tools for early prognostication have been investigated in recent years including MRI, EEG, NIRS, and clinical assessments, no tool has proven superior to all others nor to provide very consistent and highly accurate prognosis [211].

The difficulty with accurate prognostic tools remains a significant challenge as one of the most importance steps in the rehabilitation of those at risk of NDD is early identification [213]. It also remains a challenge for parents and clinicians in providing accurate counselling on the likely prognosis for the newborn, and for the implementation of future neuroprotective strategies. Several phases of injury have been identified and there is increasing recognition of ongoing injury for months and years after the HI event [18]. This provides longer therapeutic window which increases the need for early identification of those at risk of NDD and who may benefit from further interventions [214].

Various prognostic tools including serum biomarkers [106], neurophysiologic modalities such as aEEG, EEG, VEP, NIRS [307], and neuroimaging modalities have been investigated for their prognostic accuracy and provide varying degrees of prognostic accuracy [211]. Since the introduction of routine treatment of term newborns with moderate or severe NE with TH, the accuracy of these predictive tools required reassessment. In the era of routine use of TH in high-resource settings, MRI in the first week of life was the most accurate predictor of long-term outcome in NE.

MRI brain has been used since the late 1980's to predict long-term outcome in patients with NE [308]. Conventional T1/T2 weighted images were used from the earliest days and several scoring systems were developed based on these results, including Barkovich [309] and NICHD MRI scoring systems [310]. T1/T2 weighted images allow identification of signal changes indicating structural injuries following NE insult, particularly for the basal ganglia, thalami, posterior limb of the internal capsule, and motor cortex [311]. These images are best acquired between the first and second weeks of life postnatally [312]. MRI Newer methodologies including DWI and MRS have gained prominence in recent years but it is unclear whether they offer additional prognostic information compared to T1/T2 weighed images. DWI measures the diffusion of water molecules within a tissue and is restricted in areas of cell swelling indicating ischaemic injury [313]. Diffusion restriction can be quantified by the apparent diffusion coefficient (ADC). However, DWI findings pseudonormalise after the first week of life [308]. MRS measures various metabolites in brain regions that give an indication of altered brain metabolism from area peaks in



metabolites or from ratios between different metabolites. First studies of the use of MRS in NE were also conducted in the late 1980's [314], using phosphorous-MRS however MRS is not used as routinely as T1/T2 weighted imaging or DWI to date. In recent years evidence for the prognostic value of proton-MRS has suggested it may provide very powerful prognostic information of patients with NE [212]. Since brain metabolic derangements last for weeks following NE there is a wide window for obtaining MRS data.

A complication of the use of MRI as a prognostic tool in NE remains access to timing and modalities of imaging. MRI brain scans in neonates are frequently done without sedation and the time for MR image acquisition is limited, meaning there is competition between the different techniques for this time. A recent study demonstrated improved prognostic utility with MRI in the first week of life compared to the second [315], although this will depend on the MRI technique used. A further complication of MRI in to predict outcome in NE is the high incidence of incidental findings that are not associated with adverse ND outcome. A recent study reported that 47% of asymptomatic healthy term newborns have incidental findings on MRI [316] and that the majority of these findings were not associated with adverse ND outcome. Therefore, another challenge in evaluating the prognostic value of MRI is identifying specific patterns of injury associated with increased risk of death or NDD. Several tools have been validated towards this purpose including Barkovich [309], NICHD [310], Rutherford [317], and de Vries [318].

While the choice of timing and technique of MRI will depend on many factors including the clinical condition of the newborn, and access to MRI facilities we aimed to clarify the current evidence on how MRI can identify those at higher risk of death or NDD and how choice of technique may influence the usefulness of MRI in NE.

## Aims

To assess the current evidence for different MRI techniques to predict long-term outcome in neonatal encephalopathy.

## Hypothesis

Overall MRI would provide important prognostic information for patient with neonatal encephalopathy, but this varies depending on the MRI technique employed.

## Methods

Our study protocol was developed and registered with Prospero (CRD42021286461). We conducted the systematic review in accordance with the published protocol and the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) reporting guideline.

### Information sources and search strategy

4 major databases, EMBASE, MEDLINE, World of Science and the Cochrane Library were systematically searched in December 2020 to identify all relevant studies published between January 1966 and December 2020. Relevant studies were those that examined the prognostic relationship between MRI results in the first 4 weeks of life in human newborns, and neurodevelopmental outcomes  $\geq 12$  months of age. No language or publication restrictions were applied. The search strategy included the following relevant terms: 'neonatal' or 'newborn', 'neonatal encephalopathy' or 'hypoxic ischaemic encephalopathy' or 'perinatal asphyxia', 'magnetic resonance imaging' or 'MRI' or 'magnetic resonance spectroscopy' or 'MRS'. The complete search strategy and MESH terms used are reported in Appendix V, Table 33. Authors were contacted for additional details not included in the publication if they were required for inclusion in a meta-analysis. Additional data sources were sought from the references of all relevant primary studies and screening of review articles.

## Eligibility Criteria

Studies were eligible for inclusion if they reported prognostic MRI data for patients with NE over 35 weeks gestational age and neurodevelopmental outcomes at ages  $\geq 12$  months. Both observational and interventional studies were eligible for inclusion. Studies that used any clinical definition of NE, HIE, or perinatal or birth asphyxia that included features of encephalopathy in the first 24 hours of life were considered for inclusion in the quantitative analysis. To be included in a meta-analysis, studies had to report neurodevelopmental outcomes in a manner that could be dichotomised as 'normal' or 'adverse'. Studies including patients  $< 35$  weeks gestational age at birth were excluded. Both patients who received TH and those that did not were included in the study. Case reports, case-control studies, case series, in vitro studies, and animal studies were excluded.

## Predictor Definition

Any MRI technique that included MRI of the brain within the first month of life was acceptable for inclusion provided sufficient information regarding technique and timing was provided.

## Outcome Definition

The primary outcome was a 'composite' of mortality or adverse neurodevelopmental outcome at  $\geq 12$  months of age. Any clinical definition of adverse neurodevelopmental outcome including cerebral palsy, developmental delay, abnormal

neurological exam, and several standardised tests of child development including Bayley Scales of Infant Development and Griffith Scales were included.

### Screening and Selection Process

Results from the study search were uploaded to Covidence software, a software program for managing and screening literature search results, and duplicates were removed. The title, abstract and full text screening were conducted independently by 2 investigators (TH, MOD, AB, PS, MNiB, MD). Disagreements were resolved by consensus. Studies deemed eligible for inclusion were categorised by MRI technique and included in a meta-analysis.

### Bias & Quality Assessment

Risk of bias, a formal assessment of the likelihood of inaccuracy in the reported results for each individual study, for all included studies was assessed using the Quality in Prognostic Studies (QUIPS) tool [319]. Each study was assessed and assigned a rating of low, moderate, or high risk of bias according to 6 criteria: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding and statistical analysis and reporting. Assessment was completed independently by 2 researchers (TH, AB, RMcG) and disagreements resolved by consensus. Studies with  $\geq 1$  criteria at high risk of bias were excluded from the meta-analysis. Studies required all 6 categories to be at low risk of bias to be considered low risk overall. All other studies were considered to be moderate risk of bias.

## Data Extraction

A standard data extraction table was created following the CHARMS (Checklist for critical Appraisal and data extraction for systematic Reviews of prediction Modelling Studies) checklist [320]. The following data was collected: characteristics of included studies, patient characteristics, prognostic factors and outcome definitions and measurements. The data extraction form was completed independently by 2 researchers (TH, AB, PS, MD, MNiB) and disagreements were resolved by consensus. Authors were contacted by email to request data, when missing data prevented a study from inclusion in a meta-analysis.

## Data Synthesis and statistical analysis

Studies were included in a meta-analysis if they reported the prognostic factor and outcome variable in a manner that could be dichotomised. Therefore, study data was extracted as the true positive/negative and false positive/negative. Due to assumed heterogeneity between studies in participant characteristics, MRI techniques, and outcome measurement, pooled effect estimates from the meta-analyses were completed using a random effects model, DerSimonian and Laird method [321], a variation of the inverse-variance method for summarising effects across studies. The pooled dichotomous data was used to calculate the diagnostic odds ratio, sensitivity, and specificity with 95% confidence intervals. The presence of heterogeneity was assessed by visual inspection of the forest plots (a graphical representation of the results of included studies and the pooled effect estimate), and by  $\text{Chi}^2$  (a statistical test which assesses whether observed differences in

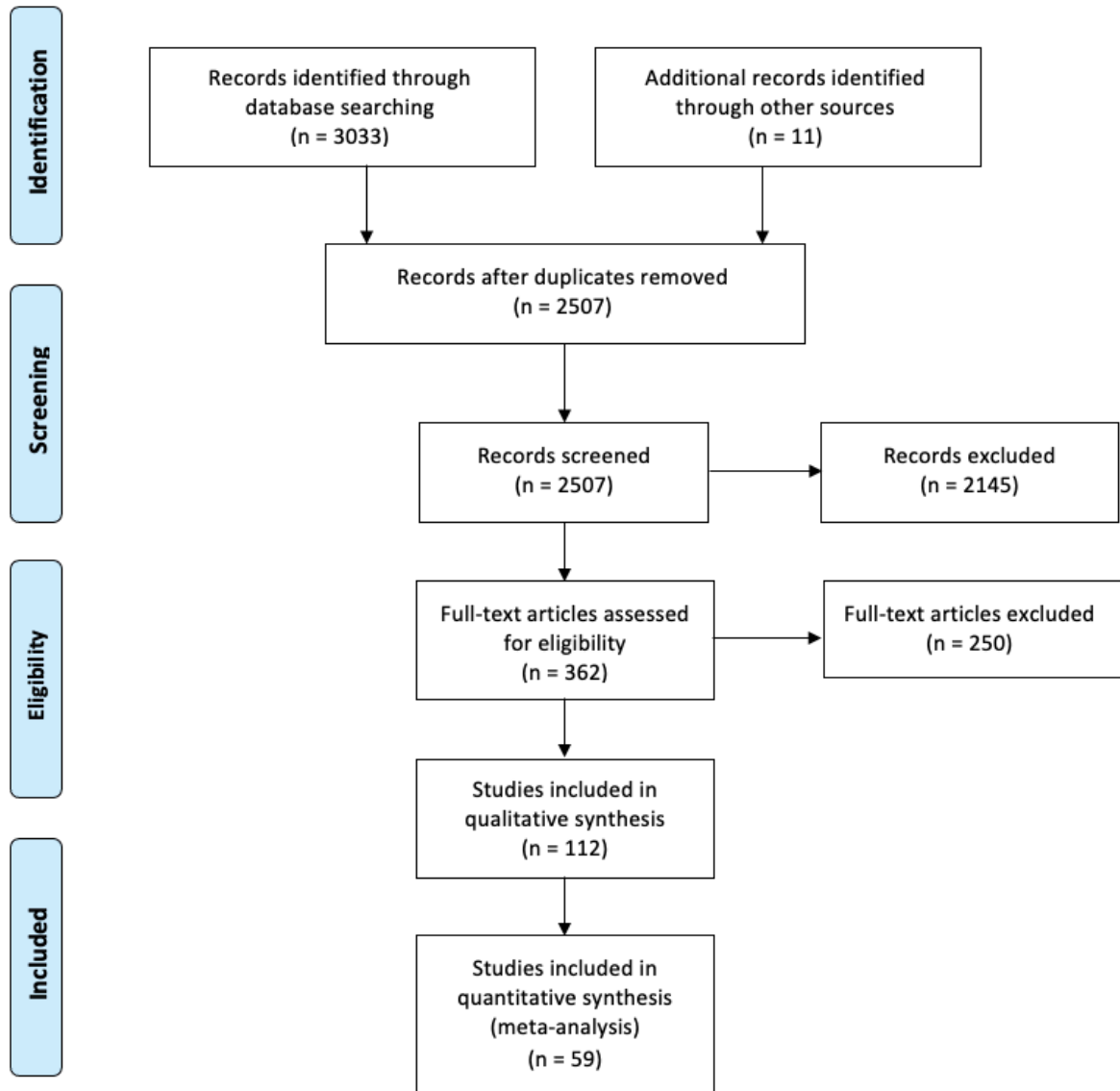
results between studies are compatible with chance alone), and  $\text{Tau}^2$  (estimate of between-study variance in random effects meta-analysis) statistics. The proportion of variance in between-study estimates attributable to heterogeneity was assessed using the  $I^2$  statistic [322]. To assess the effect of individual studies on the overall effect estimate a leave-one-out analysis was performed, an analysis of each subset of studies obtained by leaving out one study. Funnel plots, a scatter plot of the effect estimate from individual studies against the size of the study used to assess the possibility that results are missing from a meta-analysis, were created and examined to assess for small-study effects.

## Results

### Literature search and screening results

3033 studies were identified by the literature search following removal of duplicates (Figure 40). Grey literature sources provided a further 11 studies. Following title and abstract screening there were 362 studies suitable for full text screening. 112 studies were included for qualitative synthesis. From these 112 studies, 59 could be included in meta-analyses for MRI to predict long-term outcomes in NE.





*Figure 21 PRISMA flow diagram demonstrating the study selection process for this systematic review of MRI in Neonatal encephalopathy.*

## Characteristics of included studies

Articles were published between 1994 and 2020. They originated from 18 different countries. The 59 studies included in the meta-analyses included 3074 participants, ranging from 12 to 173 participants per study (Table 5) [104, 212, 310, 323-376]. Studies of design types including cohort, retrospective and prospective, case-control studies, and randomised controlled trials from which the non-intervention group could be examined independently from the intervention group were included in this systematic review. Studies were only included if both the reported MRI results and neurodevelopmental outcome results could be categorised dichotomously. All studies were conducted in a hospital setting. 48 of 59 studies (81%) included patients undergoing TH, however 11 of these 48 studies (23%) included a mixed group of patients of those treated and not treated with TH.

All studies were conducted using a 1.5T or 3T MRI scanner. 51 of 59 studies (86%) reported MRI results of conventional T1/T2 weighted imaging, 36 of 59 studies (61%) reported MRI results for diffusion weighted imaging (DWI), and 9 of 59 studies (15%) reported MRS results. It was not possible to calculate the mean or median day of MRI between all included studies due to variability in reporting methods, however all MRI studies were conducted within the first four weeks of life, and the range was from day 1 to day 28 of life. Barkovich or modified Barkovich score was the most frequently used validated MRI scoring system, reported in 22 of 59 studies (37%), followed by NICHD and Rutherford scores which were both reported in 9 of 59 studies (15%).

Similarly, it was not possible to calculate the mean or median duration of follow up across all studies, however the range was from 12 months up to 84 months of age. Adverse outcome definitions included mortality at time to follow up, formal neurodevelopmental assessment including Bayley or Griffiths or Denver developmental assessment or Wechsler Preschool and Primary Scale of Intelligence, diagnosis of cerebral palsy or epilepsy or abnormal neurological examination at the time to follow up, or any composite of these outcomes.

1st Author	Year	Country	Study Design	MRI Technique	MRI Scanner	Day of imaging	Duration to follow up	Scoring system used	Therapeutic Hypothermia
Al Amrani	2017	Canada	Retrospective cohort	T1/T2/DWI	3T	Around day 10	24 months	Barokovich	All
Al Amrani (a)	2018	Canada	Retrospective cohort	T1/T2/DWI	3T	Around day 10	24 months	Barokovich	All
Alderslieten	2011	The Netherlands	Retrospective	T1/T2/DWI/MRS	1.5T	Median day 4 (range 1-14)	18 to 46 months	Van Rooij 2010	All
Alderslieten (a)	2017	The Netherlands	Prospective cohort	T1/T2/DWI/DWI/MRS	1.5T (n = 38) 3T (n = 50)	Within 7 days	>24 months	Adapted Barokovich	All
Anzora	2013	Italy	Prospective	T1/T2/DWI/MRS	1.5T	Mean day 8.3 +/- 2.8 (SD)	24 months	Conventional imaging - Barokovich	All (selective head)
Ayrapetyan	2018	USA	Prospective cross-sectional	T1/T2	Not specified	Median day 8 (IQR 7-12)	Range 4 months - 5 years	Barokovich	All
Azopardi	2019	UK	RCT	T1/T2/DWI/MRS	3T	Within 15 days	Median 25 months (IQR 24-29)	Rutherford (TOBVI) score (not used here)	All
Badi	2014	UK	Cohort	MRS	3T	Postnatal age at scan 5.6 +/- 1.7 days (mean +/- SD)	12 months	None specified	Not specified
Barta	2018	Hungary	Prospective cohort	T1/T2/DWI/MRS	3T	Median 25th hour (range 6-96)	18-26 months	None specified	All
Barta (a)	2002	Hungary	Retrospective cohort	MRS	3T	Median day 4 (range 1-14)	18-26 months	None specified	All
Balet	2002	Turkey	Prospective	T1/T2	Not specified	Median day 11 (range 5-19)	3.5-4 years of age	Grossman	Not specified
Baloch	2006	France	Retrospective cohort	T1/T2/DWI/MRS	1.5T	Mean day 6 (range 2-12)	Minimum 12 months	None specified	Not specified
Chalikh	2018	USA	Prospective cohort	T1/T2/DWI	Not specified	<30 days of age	Mean 19 months (SD)	NICHD	None
Chang	2020	Korea	Retrospective	T1/T2/DWI	3T	Within 10 days	18-24 months	NICHD	All
Charon	2016	France	Retrospective cohort	T1/T2/DWI	1.5T	Median day 4 (range 3-6)	Median 24/12 (range 18-41)	Own & Barokovich	All
Cheung	2012	Australia	RCT substudy	T1/T2/DWI	1.5 or 3T	Median day 6 (IQR 3-8)	24 months	Rutherford	66 TH/ 61 NT
Chiang	2016	Taiwan	Prospective cohort	T1/T2/DWI	Not specified	Between day 7 & 14	18 months	None specified	All
Chiang	2016	Taiwan	Prospective cohort	T1/T2	Not specified	Mean day 4.6 +/- 1.3 (SD)	24 months	Barokovich	24 TH/ 4 NT
De Vis	2015	The Netherlands	Retrospective cohort	T1/T2/DWI	3T	Median day 5 (IQR 4-6)	24 months	Adapted Barokovich	45 TH/ 37 NT
De Wisselare	2019	The Netherlands	Retrospective cohort	T1/T2/DWI/MRS/ASL	1.5T	Median day 11 (IQR 8-14)	Median 24 months (IQR 24-24)	Rutherford	All
Dunne	2016	UK	Retrospective cohort	T1/T2/DWI	1.5T	Median day 15 (range 8-25)	18 months	Barokovich	None
El-Ayouty	2007	Egypt	Prospective cohort	T1/T2	1.5T	Mean day 10.32 (+/- 1.78)	12 months	None specified	All
El Behairy	2019	Egypt	Prospective cross-sectional	T1/T2	1.5T	5th-7th DOL	6-24 months	De Vries 2010 classification	All
Goerke	2018	USA	Retrospective cohort	T1/T2/DWI/DWI	Not specified	Day 3-8	24 to 26 months	Griffiths	None
Goergen	2014	Australia	Prospective cohort	T1/T2/DWI/FLAIR	1.5T	Median day 5	18 months	Barokovich	All
Gulliot	2019	Canada	Retrospective cohort	T1/T2/DWI/MRS	1.5 or 3T	Median day 6 (range 2-42) (88% within 10/7)	42 months	Barokovich	All
Hayes	2016	Ireland	Mixed retrospective & prospective	T1/T2/DWI/MRS	1.5T	Day 4-6	12-24 months	None specified	All
Heussen	2017	Spain	Prospective cohort	T2/DWI	1.5T	5-14 days of life	12 months	Barokovich	All
Jordan-Crowe	2019	Spain	Retrospective cohort	T1/T2	Not specified	Day 7-10	24 months	None specified	None
Jyoti	2006	Australia	Prospective cohort	T1/T2/DWI/FLAIR	1.5T	Day 1-2	Mean 2.6 years +/- 1.2 (SD)	Own scoring system	None
Kalay	2011	Turkey	Retrospective cohort	T1/T2/DWI	1.5T	Mean day 6.7 (range 2-13)	18-24 months	None specified	Not specified
Khong	2004	China	Prospective cohort	T1/T2/DWI/MRS	1.5T	Mean day 6.1 (range 1-14)	Mean 18.6 months (range 6-30)	None specified	None
Kuencie	1994	Switzerland	Prospective cohort	T1/T2/DWI	2.35T	Day 1-2	Mean 18.6 months (range 6-30)	Own classification system	None
L'Abbe	2005	The Netherlands	Prospective cohort	T1/T2	1.5T	Day 1-2	24 months	None specified	None
Lakatos	2019	Hungary	Retrospective	T1/T2/DWI/MRS	1.5T	Within 1 week (65.12 +/- 46.34 h)	18-26 months	Barokovich	All
Lally	2019	UK & USA	Prospective cohort	T1/T2/DWI/MRS	3T	Mean age of 8.4 days (SD 4.3)	Median 23 months (IQR 20-25)	None specified	All
Leon-Locano	2020	Spain	Prospective cohort	T1/T2/DWI/MRS	3T	Median day 10 (IQR 4.4, 13.9)	24 months	Rutherford	All
Li	2013	Japan	Prospective cohort	T1/T2/DWI	1.5T	End of the second postnatal week	At least 3 years (mean 5 +/- 1.6 yrs)	Modified Okerefor	All
Marchiò	2018	Italy	Retrospective cohort	T1/T2	1.5T	5.7 +/- 3.7 days	24 months	Bednarek severity scores	All
Mastrangelo	2019	Italy	Retrospective cohort	T1/T2/DWI	1.5T	7-10 days	24 months	None specified	All
McDonagh	2017	USA	Retrospective cohort	T1/T2/DWI/MRS	1.5T	Between 1 and 4 weeks of life	24 months	Own classification system	All (selective head)
Mercuri	2000	UK	Prospective cohort	T1/T2/DWI	1T	Median day 5 (range 2-14); restricted here day 2-9	18-24 months	NICHD	All
Mitra	2019	UK	Retrospective service improvement	T1/T2/DWI/MRS	Not specified	Postnatal age range 7-28 of life	18-22 months	CHONG	All
Monani	2018	Italy	Retrospective	T1/T2/DWI	1.5T	Between day 5 (range 2-14); restricted here day 2-9	24 months	Modified Barokovich & Mercuri	8/14 had TH
Parikh	2009	USA	RCT substudy	T1/T2/DWI	1.5T	Postnatal age range 38.6 - 45.9 weeks	4 years	NICHD and Barokovich	None
Reiss	2019	USA	Retrospective	T1/T2/DWI	1.5T	Mean day 7 (range 5-14)	24 months	NICHD	All
Rollins	2014	USA	Retrospective cohort	T1/T2/FLAIR/DWI	1.5T or 3T	Mean day 7 (range 5-14)	18 months	NICHD	64 TH/67 NT
Rutherford	2014	USA	RCT substudy (TOBVI)	T1/T2	1.5 or 3T	Within the first 4 weeks	18 months	NICHD	73 TH/63 NT
Shankaran	2012	USA	RCT follow up	T1/T2	1.5 or 3T	Mean day 15 +/- 12 (SD)	18-22 months	NICHD	61 TH/7 NT
Shibasaki	2018	Japan	Retrospective	T1/T2/MRS	3T	18-36 hours (n = 25) and 7-14 days (n = 64) after birth	24 months	None specified	All
Skranes	2017	Norway	Prospective	T1/T2/DWI	1.5 or 3T	Day 4-5 & 10-11 (later used if available)	24 months	Rutherford (TOBVI) score & Bonifacio (mc AI)	All
Sweetman	2020	Ireland	Prospective	T1/T2	1.5T	Median day 7 (IQR 7-10)	24 months	Barokovich	All
Tharapooopathy	2020	UK	Prospective (BIBINS)	T1/T2	1.5T	Median age of 6 (IQR: 7-11) days	Minimum of 18 months	Rutherford	All
Trivedi	2017	USA	Prospective	T1/T2/DWI	1.5 or 3T	Scan 1 within 1st 6 days, scan 2 between day 7-14	18-24 months	Own scoring, Bednarek	All
Tusor	2012	UK	Retrospective cohort	T1/T2/DWI	3T	Median day 5 (range 2-10)	Median 24 months (range 12 - 28)	Rutherford	All
Townley	2010	Ireland	Retrospective cohort	T1/T2/DWI	1.5T	Median day 5 (range 2-10)	24 months	Barokovich	Not specified
Van Schie	2015	The Netherlands	Retrospective cohort	T1/T2/DWI/FLAIR	Not specified	Within the first week of life	7 years	Barokovich	None
Weekle	2016	The Netherlands	RCT substudy (MEMO)	T1/T2/DWI	1.5 or 3T	Mean age of 6.4 days (SD 2.9) mean postmenstrual	24 months	Van Rooij 2010 (modified Barokovich)	All
Weekle	2018	The Netherlands	Retrospective cohort	T1/T2/DWI + MRS in cohort 1	1.5 or 3T	Day 6 (5-8) Majority of scans were obtained in the first 24 months	Median age at last follow-up was: Modified Barokovich	Own score (de Vries)	All
Xu	2019	Canada	Retrospective cohort	T1/T2/DWI	1.5T	Between days 3 and 5 of life	Median age at last follow-up was: Modified Barokovich	Modified Barokovich	30% (n=55)

Table 5 Characteristics of included studies.

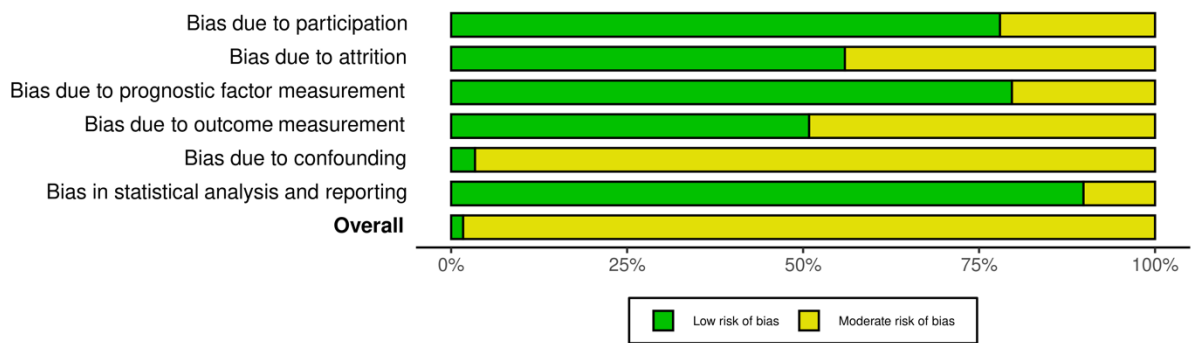
## Risk of bias in included studies

We reviewed risk of bias for each included study for the meta-analysis of the total MRI and did not re-evaluate each study for the separate meta-analyses as there was no significant difference in methodology between MRI or outcome measurement between the different MRI techniques.

The risk of bias for each included study was assessed using the QUIPS tool. Each study was assessed by study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. A study was judged to be overall high risk of bias if 1 or more domains were judged to be at high risk of bias. We intended to exclude any study that was judged to be high risk of bias, however this was not required for the current review. A study was judged to be overall low risk of bias if it was considered to be low risk in all 6 domains. Only one study was considered overall low risk of bias, and all other included studies were considered overall moderate risk of bias (Figure 22) [104, 212, 310, 323-376].



**Figure 22 Risk of bias traffic light plot for studies included in the systematic review of MRI to predict outcome in neonatal encephalopathy. This plot was generating using robvis software developed by McGuinness et al [377].**



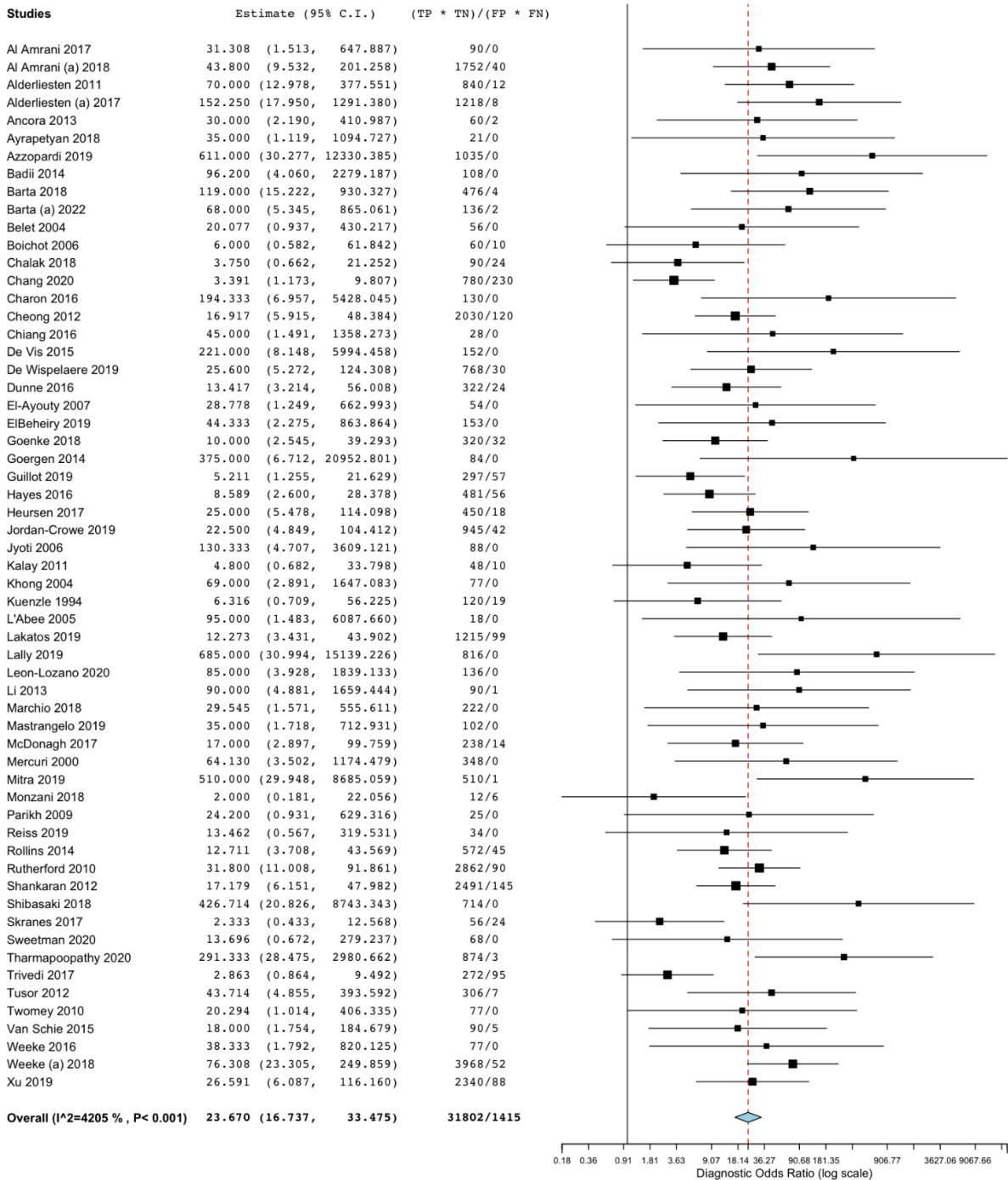
**Figure 23 Risk of bias summary plot for studies included in the systematic review of MRI to predict outcome in neonatal encephalopathy.** This plot was generating using robvis software developed by McGuinness et al [377].

## MRI to predict outcome in NE, all techniques included

Overall, the composite of studies of all MRI techniques included in this systematic review demonstrated that an abnormal MRI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 23.7 (95% CI 16.7 to 33.5) compared to those with normal MRI brain (Figure 24) [104, 212, 310, 323-376]. The meta-analysis included 3092 participants from 59 studies. Results were consistent across many studies, and abnormal MRI brain was associated with significantly increased OR of adverse outcome in 48 of 59 included studies. There was not conclusive evidence in the remaining 11 studies of a statistically significant relationship, and no study reported an association between abnormal MRI brain and decreased OR of adverse long-term outcome.

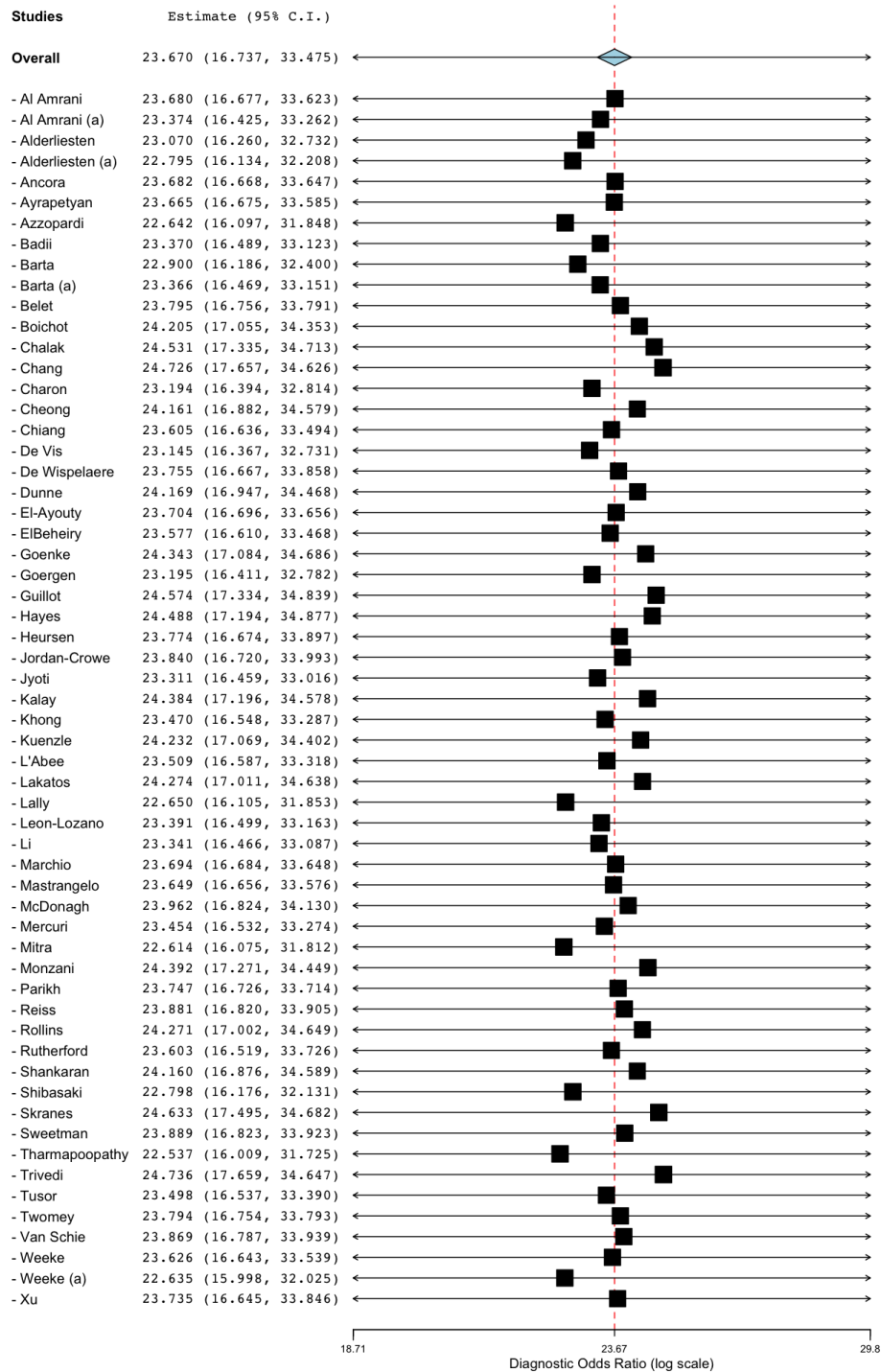
There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  100.1, df 58). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was moderate ( $I^2$  42.1%, Tau 0.7). This likely reflects variation between studies on severity of NE in patients included, differences between studies regarding treatment with therapeutic hypothermia, duration of time to follow up, and variation in MRI technique and timing.





**Figure 24 Odds ratio (OR) of all MRI techniques to predict long-term outcome in neonatal encephalopathy. An abnormal MRI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 23.7 (95% CI 16.7 to 33.5) compared to those with normal MRI brain.**

A sensitivity analysis was conducted using the leave-one-out method. Although some studies reported odds ratios that differed significantly from the composite OR, none were significantly different or carried sufficient weight to alter the composite OR independently (Figure 25) [104, 212, 310, 323-376]. See Table 6 [104, 212, 310, 323-376] for further details.



**Figure 25** Leave-one-out forest plot, total MRI. No individual study had a significant effect alone on the overall results of the meta-analysis.

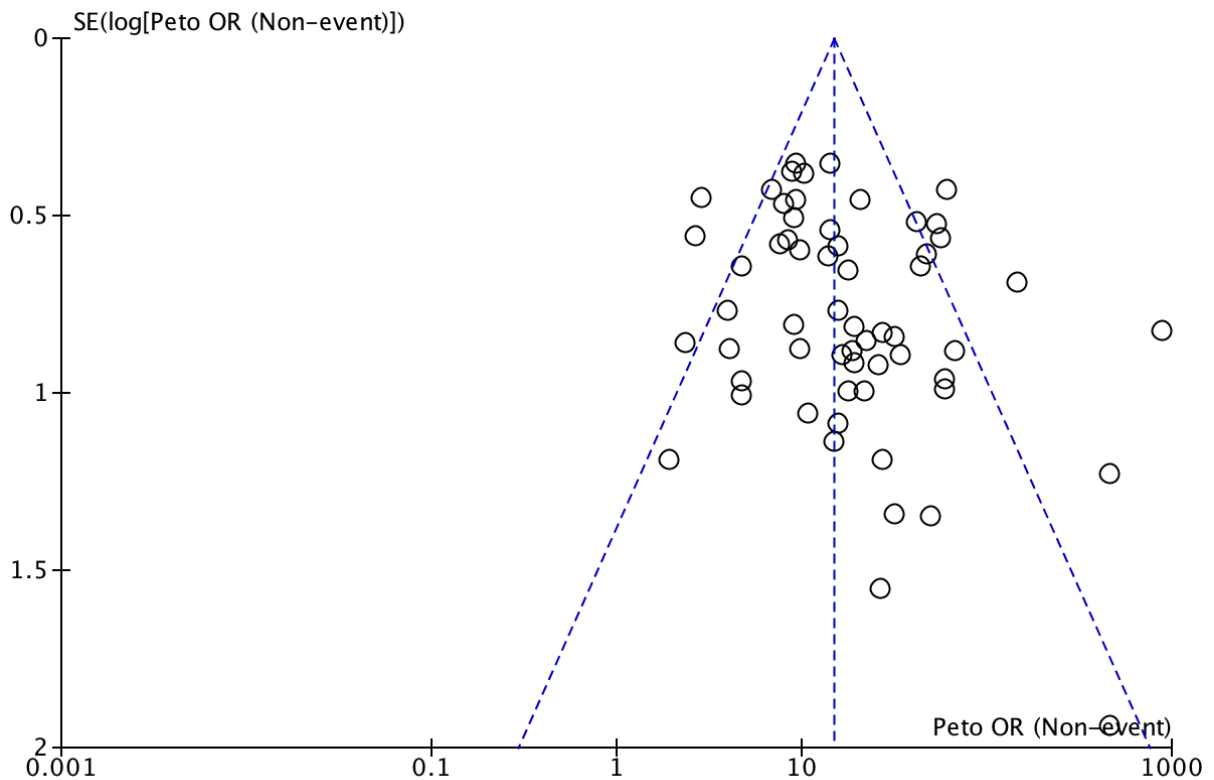
Diagnostic Random-Effects Model  
Metric: Diagnostic Odds Ratio

Model Results

Studies	Estimate	Lower bound	Upper bound	Std. error	p-Val
Overall	20.864	14.780	29.452	0.176	< 0.001
- Al Amrani	20.842	14.707	29.535	0.178	< 0.001
- Al Amrani (a)	20.517	14.453	29.124	0.179	< 0.001
- Alderliesten	21.252	14.916	30.280	0.181	< 0.001
- Alderliesten (a)	21.515	15.239	30.375	0.176	< 0.001
- Ancora	20.836	14.694	29.544	0.178	< 0.001
- Ayrapetyan	20.834	14.709	29.510	0.178	< 0.001
- Azzopardi	19.924	14.202	27.954	0.173	< 0.001
- Badi	20.568	14.543	29.090	0.177	< 0.001
- Barta	20.122	14.259	28.395	0.176	< 0.001
- Barta (a)	20.552	14.518	29.095	0.177	< 0.001
- Belet	20.945	14.777	29.687	0.178	< 0.001
- Boichot	21.302	15.033	30.185	0.178	< 0.001
- Chalak	21.590	15.274	30.519	0.177	< 0.001
- Chang	21.797	15.556	30.543	0.172	< 0.001
- Charon	20.415	14.462	28.819	0.176	< 0.001
- Cheong	21.206	14.843	30.296	0.182	< 0.001
- Chiang	20.780	14.674	29.427	0.178	< 0.001
- De Vis	20.372	14.438	28.745	0.176	< 0.001
- De Wispelaere	20.865	14.670	29.677	0.180	< 0.001
- Dunne	21.236	14.916	30.236	0.180	< 0.001
- El-Ayouty	20.865	14.724	29.567	0.178	< 0.001
- ElBeheiry	20.749	14.647	29.394	0.178	< 0.001
- Goenke	21.395	15.036	30.444	0.180	< 0.001
- Goergen	20.424	14.482	28.804	0.175	< 0.001
- Guillot	21.620	15.264	30.622	0.178	< 0.001
- Hayes	21.526	15.130	30.626	0.180	< 0.001
- Heursen	20.879	14.675	29.707	0.180	< 0.001
- Jordan-Crowe	20.940	14.716	29.798	0.180	< 0.001
- Jyoti	20.519	14.518	28.999	0.176	< 0.001
- Kalay	21.457	15.152	30.386	0.178	< 0.001
- Khong	20.657	14.595	29.237	0.177	< 0.001
- Kuenzle	21.323	15.043	30.225	0.178	< 0.001
- L'Abbe	20.702	14.637	29.282	0.177	< 0.001
- Lakatos	21.561	15.214	30.555	0.178	< 0.001
- Lally	19.933	14.210	27.962	0.173	< 0.001
- Leon-Lozano	20.586	14.551	29.123	0.177	< 0.001
- Li	20.538	14.520	29.051	0.177	< 0.001
- Marchio	20.852	14.712	29.555	0.178	< 0.001
- Mastrangelo	20.813	14.688	29.492	0.178	< 0.001
- McDonagh	21.062	14.815	29.944	0.180	< 0.001
- Mercuri	20.639	14.578	29.218	0.177	< 0.001
- Mitra	19.896	14.180	27.916	0.173	< 0.001
- Monzani	21.480	15.229	30.297	0.175	< 0.001
- Parikh	20.904	14.752	29.622	0.178	< 0.001
- Reiss	21.023	14.834	29.794	0.178	< 0.001
- Rollins	21.321	14.958	30.392	0.181	< 0.001
- Rutherford	20.686	14.517	29.478	0.181	< 0.001
- Shankaran	21.203	14.837	30.301	0.182	< 0.001
- Shibasaki	20.062	14.269	28.205	0.174	< 0.001
- Skranes	21.694	15.420	30.521	0.174	< 0.001
- Sweetman	21.028	14.835	29.806	0.178	< 0.001
- Tharmapooopathy	19.812	14.113	27.812	0.173	< 0.001
- Trivedi	21.802	15.562	30.544	0.172	< 0.001
- Tusor	20.660	14.571	29.294	0.178	< 0.001
- Twomey	20.943	14.774	29.688	0.178	< 0.001
- Van Schie	20.996	14.794	29.799	0.179	< 0.001
- Weeke	20.794	14.677	29.460	0.178	< 0.001
- Weeke (a)	19.814	14.056	27.932	0.175	< 0.001
- Xu	20.841	14.647	29.655	0.180	< 0.001

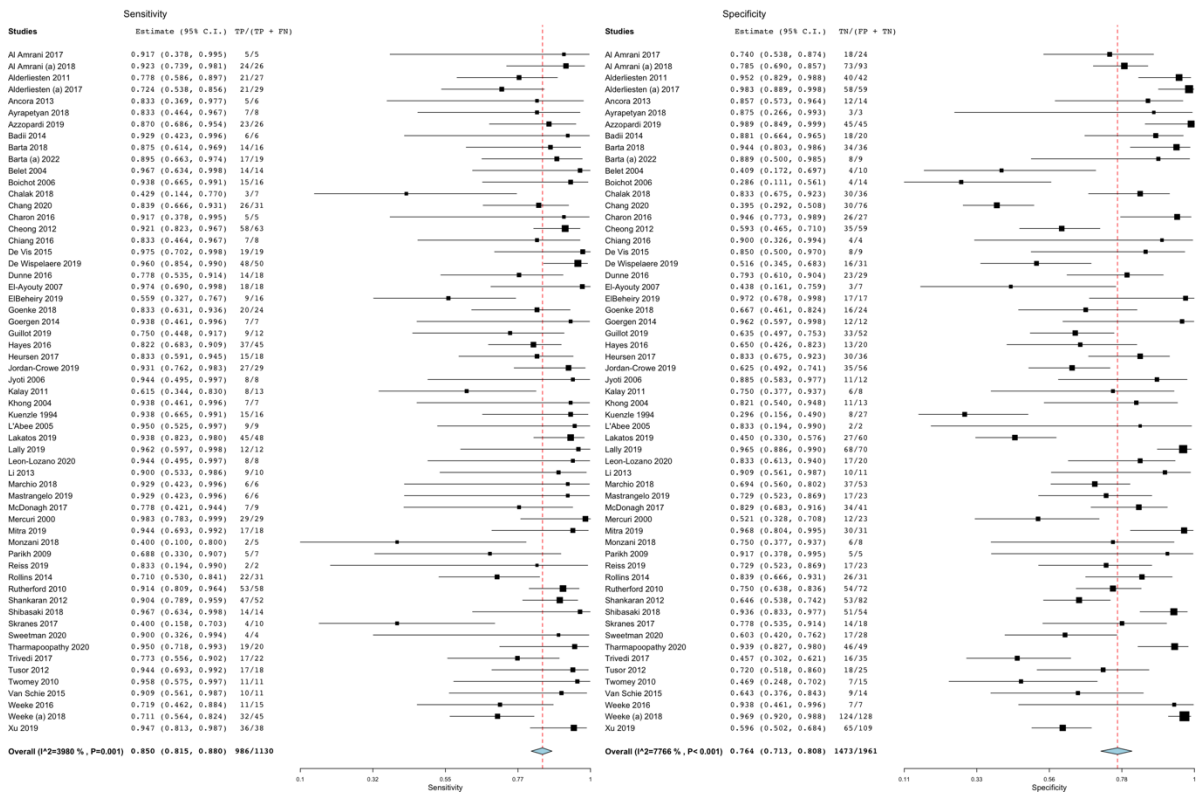
**Table 6 Results of leave-one-out analysis for all MRI studies to predict long-term outcome in neonatal encephalopathy.**

Visual inspection of the funnel plot did not demonstrate significant asymmetry to suggest small study bias was a significant issue with this systematic review (Figure 26). The largest included studies are close to the summary estimate, and although some studies lie outside of the 95% confidence intervals, they are distributed on either side of the summary estimate line with a slight predominance for small studies to the right of the summary effect line, indicating higher OR than the effect estimate. Unfortunately, neither the software OpenMetaAnalyst nor RevMan provide for statistical investigation of the effect of small study bias.



**Figure 26** Funnel plot of all included studies of MRI to predict long-term outcome in neonatal encephalopathy. No significant issue with small study bias was evident.

Overall, MRI brain regardless of technique, was associated with a sensitivity of 85% (95% CI 81.5 to 88) to predict adverse long-term outcome ( $\text{Chi}^2$  96, df 58,  $I^2$  39.8%), and a specificity of 76.4% (95% CI 71.3 to 80.8) to predict adverse long-term outcome in NE ( $\text{Chi}^2$  259.6, df 58,  $I^2$  77.7%) (Figure 27) [104, 212, 310, 323-376]. This suggests that MRI brain is better at identifying those at higher risk of adverse outcome to provide reassurance for those that are not at increased risk of adverse outcome. This may also reflect the choice the cut-off values chosen by researchers which may have been deliberately favourable towards inclusion of patients at unclear risk of adverse outcome, as they may have had a preference towards identifying patients at risk rather than eliminating those at lower risk. This may also reflect the variation in participants included in each study. For example, although the reverse of the overall trend, the study by Chalak 2018 [378] only included patients with mild NE, therefore the MRI results were always likely to provide higher specificity than sensitivity results.

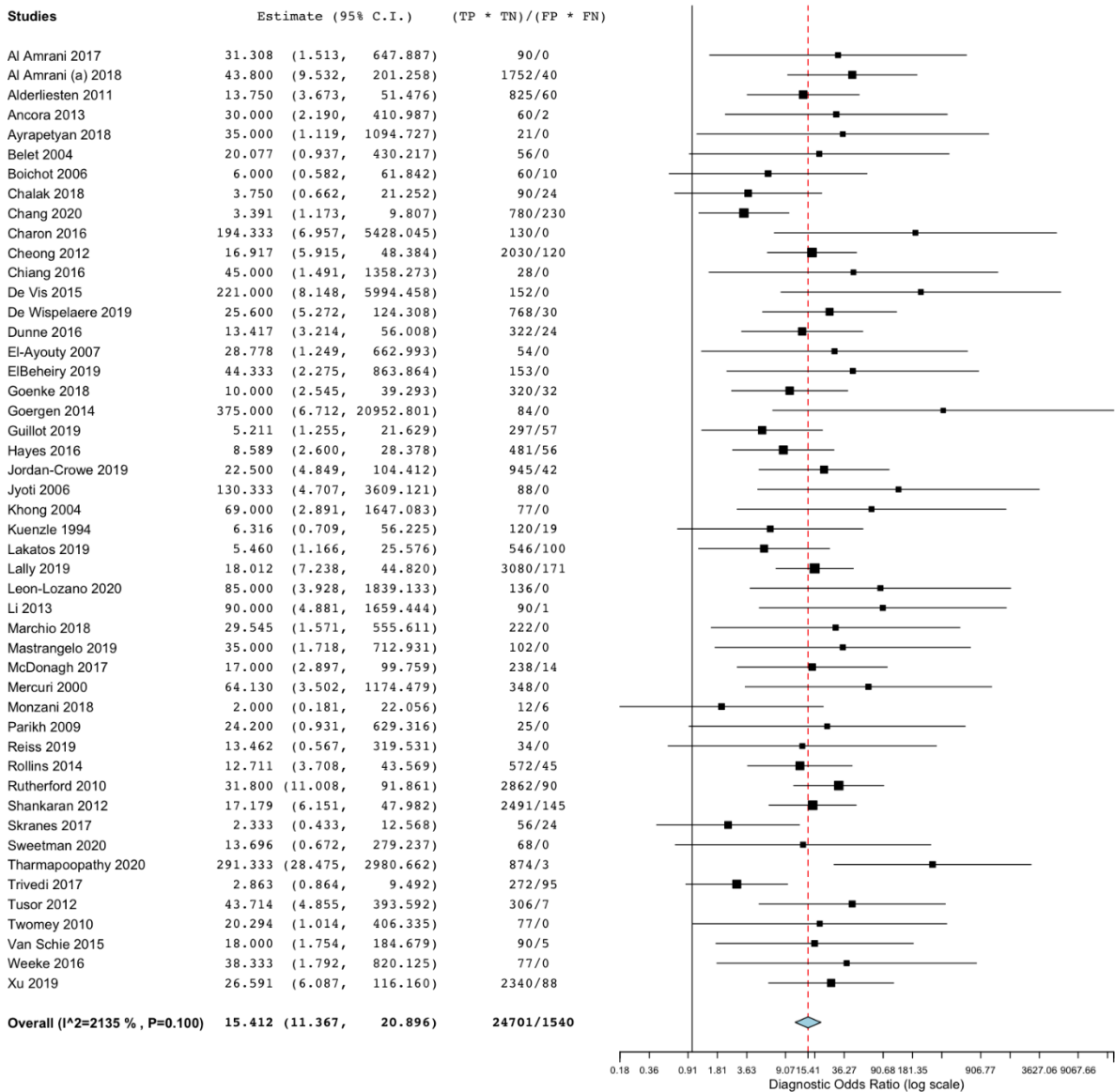


**Figure 27 Sensitivity and specificity of any MRI technique to predict long-term outcome in neonatal encephalopathy.** MRI brain regardless of technique, was associated with a sensitivity of 85% (95% CI 81.5 to 88) to predict adverse long-term outcome ( $\text{Chi}^2$  96, df 58,  $I^2$  39.8%), and a specificity of 76.4% (95% CI 71.3 to 80.8) to predict adverse long-term outcome in NE ( $\text{Chi}^2$  259.6, df 58,  $I^2$  77.7%).

## MRI to predict outcome in NE, conventional T1/T2 weighted imaging

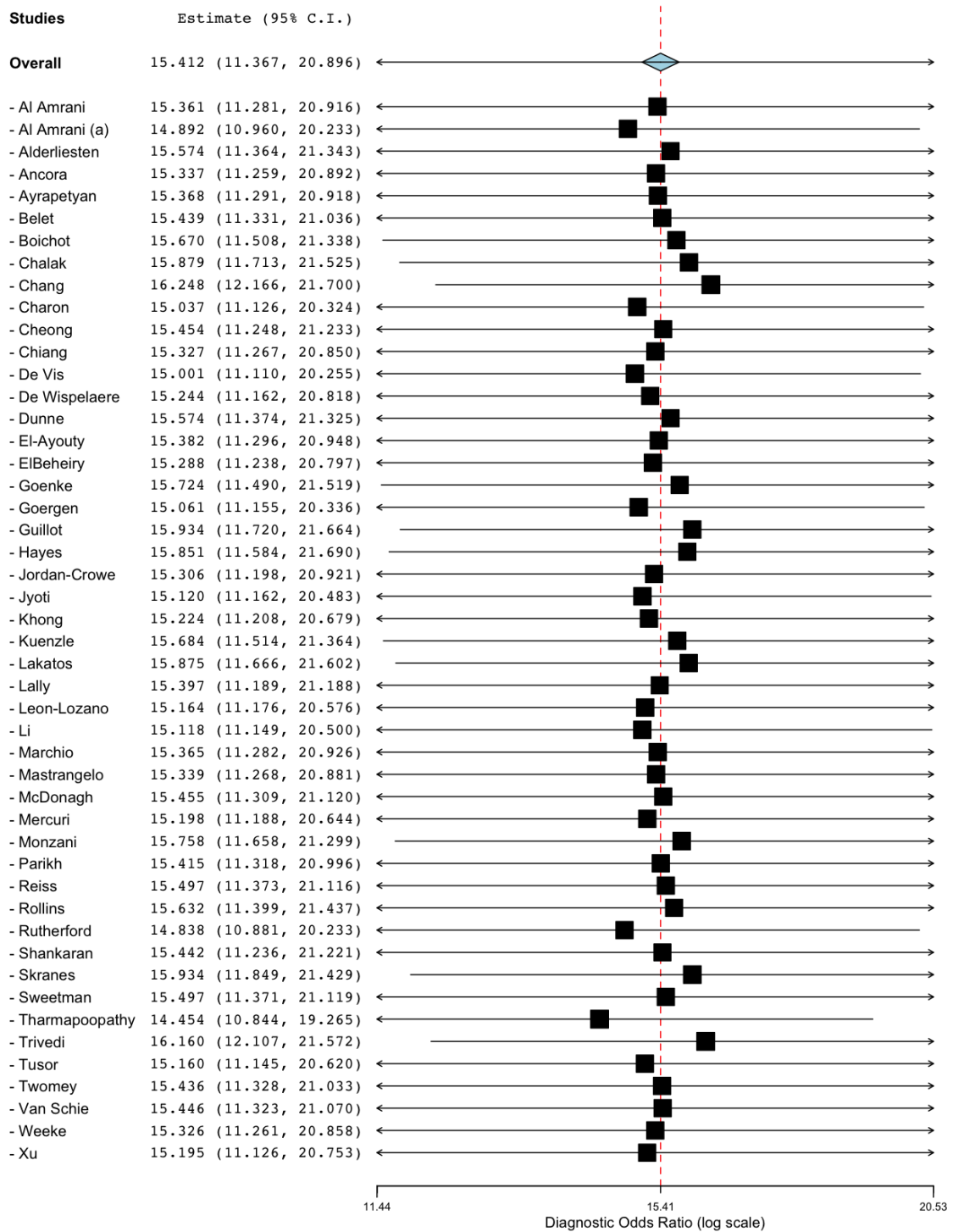
Overall, the composite of studies of conventional T1/T2 weighted MRI techniques included in this systematic review demonstrated that an abnormal T1/T2 weighted MRI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 15.4 (95% CI 11.4 to 20.9) compared to those with normal MRI brain (Figure 28) [104, 212, 310, 323-325, 327, 328, 331-346, 348, 349, 351, 352, 354-360, 362-366, 368-373, 375, 376]. The meta-analysis included 2559 participants from 49 studies. Results were consistent across many studies, and abnormal MRI brain was associated with significantly increased OR of adverse outcome in 10 of 49 included studies. There was not conclusive evidence in the remaining studies of a statistically significant relationship, and no study reported an association between abnormal MRI brain and decreased OR of adverse long-term outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  59.8, df 48). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was low ( $I^2$  21.3%, Tau 0.2). This likely reflects variation between studies on severity of NE in patients included, differences between studies regarding treatment with therapeutic hypothermia, duration of time to follow up, and variation in MRI technique and timing. Lower between study variability may be attributable to less significant effect of timing on T1/T2 MRI results.





**Figure 28** Odds ratio of any conventional T1/T2 weighted MRI technique to predict long-term outcome in neonatal encephalopathy. Abnormal T1/T2 weighted MRI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 15.4 (95% CI 11.4 to 20.9) compared to those with normal MRI brain.

A sensitivity analysis was conducted using the leave-one-out method. Although some studies reported odds ratios that differed significantly from the composite OR, none were significantly different or carried sufficient weight to alter the composite OR independently (Figure 29) [104, 212, 310, 323-325, 327, 328, 331-346, 348, 349, 351, 352, 354-360, 362-366, 368-373, 375, 376]. See Table 7 [104, 212, 310, 323-325, 327, 328, 331-346, 348, 349, 351, 352, 354-360, 362-366, 368-373, 375, 376] for further details.



**Figure 29** Leave-one-out forest plot for studies including T1/T2 weighted MRI results. No individual study had a significant effect alone on the overall results of the meta-analysis.

Diagnostic Random-Effects Model

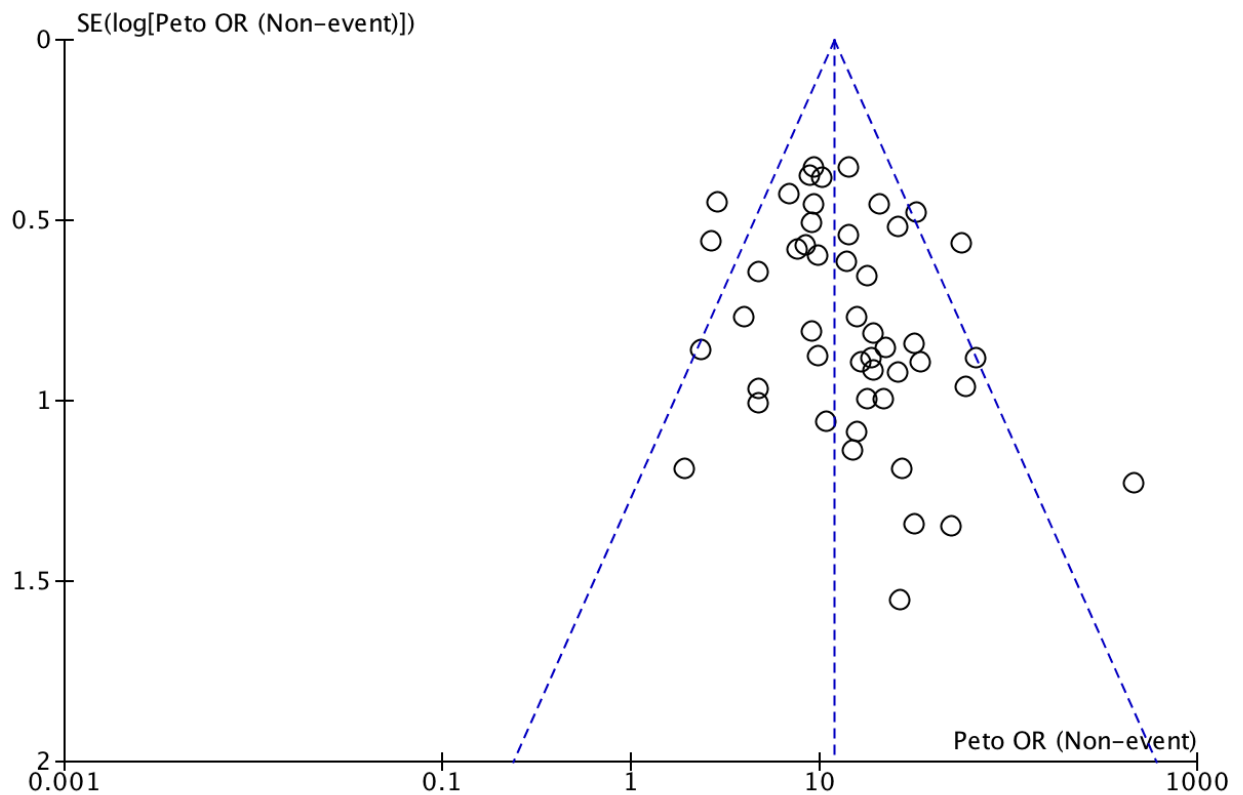
Metric: Diagnostic Odds Ratio

Model Results

Studies	Estimate	Lower bound	Upper bound	Std. error	p-Val
Overall	16.345	11.714	22.808	0.170	< 0.001
- Al Amrani	16.296	11.625	22.844	0.172	< 0.001
- Al Amrani (a)	15.814	11.291	22.148	0.172	< 0.001
- Alderliesten	16.585	11.747	23.415	0.176	< 0.001
- Ancora	16.274	11.603	22.825	0.173	< 0.001
- Ayrapetyan	16.301	11.635	22.840	0.172	< 0.001
- Azzopardi	15.397	11.189	21.188	0.163	< 0.001
- Belet	16.389	11.685	22.988	0.173	< 0.001
- Boichot	16.673	11.890	23.378	0.172	< 0.001
- Chalak	16.910	12.104	23.623	0.171	< 0.001
- Chang	17.188	12.462	23.707	0.164	< 0.001
- Charon	15.903	11.423	22.140	0.169	< 0.001
- Cheong	16.483	11.642	23.337	0.177	< 0.001
- Chiang	16.252	11.605	22.759	0.172	< 0.001
- De Vis	15.860	11.402	22.060	0.168	< 0.001
- De Wispelaere	16.206	11.518	22.801	0.174	< 0.001
- Dunne	16.580	11.754	23.386	0.175	< 0.001
- El-Ayouty	16.321	11.642	22.880	0.172	< 0.001
- ElBeheiry	16.209	11.573	22.703	0.172	< 0.001
- Goenke	16.743	11.875	23.605	0.175	< 0.001
- Goergen	15.926	11.453	22.148	0.168	< 0.001
- Guillot	16.962	12.105	23.767	0.172	< 0.001
- Hayes	16.876	11.969	23.796	0.175	< 0.001
- Jordan-Crowe	16.278	11.560	22.923	0.175	< 0.001
- Jyoti	16.003	11.471	22.326	0.170	< 0.001
- Khong	16.130	11.533	22.560	0.171	< 0.001
- Kuenzle	16.690	11.898	23.411	0.173	< 0.001
- Lakatos	16.902	12.054	23.700	0.172	< 0.001
- Leon-Lozano	16.059	11.493	22.439	0.171	< 0.001
- Li	16.007	11.462	22.354	0.170	< 0.001
- Marchio	16.303	11.628	22.858	0.172	< 0.001
- Mastrangelo	16.270	11.609	22.802	0.172	< 0.001
- McDonagh	16.433	11.676	23.129	0.174	< 0.001
- Mercuri	16.102	11.512	22.523	0.171	< 0.001
- Monzani	16.781	12.050	23.369	0.169	< 0.001
- Parikh	16.359	11.668	22.936	0.172	< 0.001
- Reiss	16.459	11.735	23.085	0.173	< 0.001
- Rollins	16.651	11.786	23.526	0.176	< 0.001
- Rutherford	15.836	11.262	22.269	0.174	< 0.001
- Shankaran	16.473	11.632	23.330	0.178	< 0.001
- Skranes	16.969	12.235	23.534	0.167	< 0.001
- Sweetman	16.460	11.733	23.091	0.173	< 0.001
- Tharmapooopathy	15.224	11.068	20.941	0.163	< 0.001
- Trivedi	17.140	12.434	23.625	0.164	< 0.001
- Tusor	16.076	11.474	22.522	0.172	< 0.001
- Twomey	16.386	11.682	22.985	0.173	< 0.001
- Van Schie	16.408	11.682	23.046	0.173	< 0.001
- Weeke	16.254	11.601	22.774	0.172	< 0.001
- Xu	16.160	11.483	22.742	0.174	< 0.001

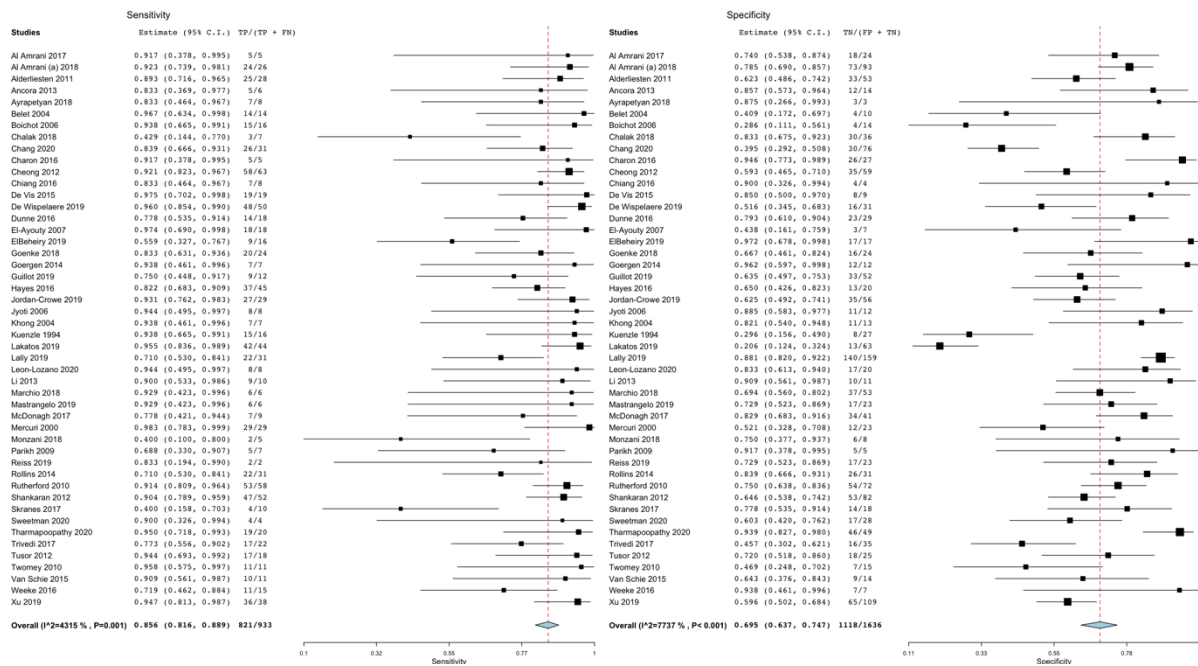
**Table 7 Results of leave-one-out analysis for T1/T2 weighted MRI studies to predict long-term outcome in neonatal encephalopathy.**

Visual inspection of the funnel plot did not demonstrate significant asymmetry to suggest small study bias was a significant issue with this systematic review (Figure 30). The largest included studies are close to the summary estimate, and although some studies lie outside of the 95% confidence intervals, they are distributed equally on either side of the summary estimate line.



**Figure 30** Funnel plot of included studies for T1/T2 weighted imaging to predict long-term outcome in neonatal encephalopathy. No significant issue with small study bias was evident.

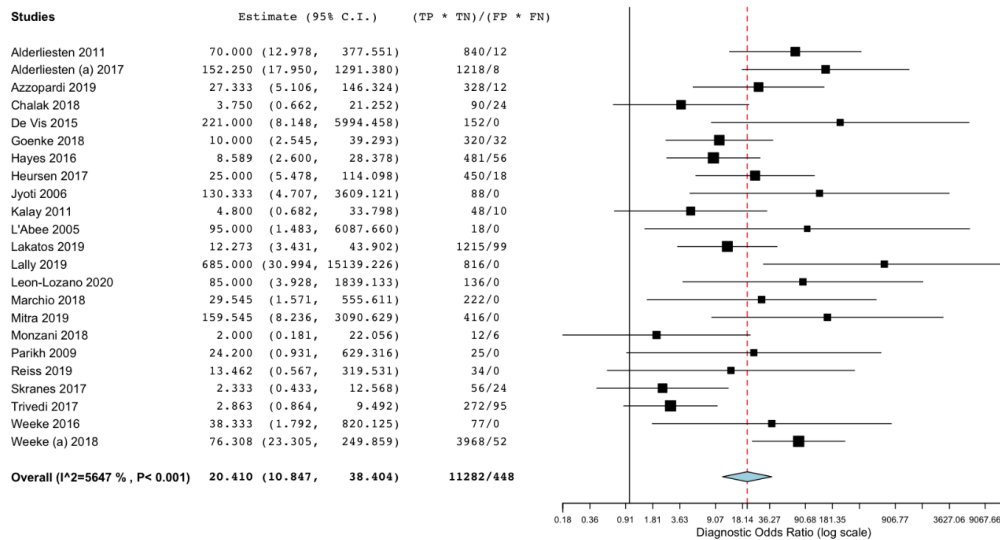
T1/T2 weighted MRI brain was associated with a sensitivity of 85.6% (95% CI 81.6 to 88.9) to predict adverse long-term outcome (Chi<sup>2</sup> 82.7, df 48, I<sup>2</sup> 43.2%), and a specificity of 69.5% (95% CI 63.7 to 74.7) to predict adverse long-term outcome in NE (Chi<sup>2</sup> 207.7, df 48, I<sup>2</sup> 77.4%) (Figure 31) [104, 212, 310, 323-325, 327, 328, 331-346, 348, 349, 351, 352, 354-360, 362-366, 368-373, 375, 376]. These results closely resemble the overall MRI results, which is unsurprising given the high proportion of overall included studies that reported results for T1/T2 weighted MRI. This suggests, as with the overall MRI results, that MRI brain is better at identifying those at higher risk of adverse outcome to provide reassurance for those that are not at increased risk of adverse outcome.



**Figure 31 Sensitivity and specificity of T1/T2 weighted MRI technique to predict long-term outcome in neonatal encephalopathy.** T1/T2 weighted MRI brain was associated with a sensitivity of 85.6% (95% CI 81.6 to 88.9) to predict adverse long-term outcome (Chi<sup>2</sup> 82.7, df 48, I<sup>2</sup> 43.2%), and a specificity of 69.5% (95% CI 63.7 to 74.7) to predict adverse long-term outcome in NE (Chi<sup>2</sup> 207.7, df 48, I<sup>2</sup> 77.4%).

## MRI to predict outcome in NE, diffusion weighted imaging

The composite of studies of diffusion weighted imaging (DWI) MRI techniques included in this systematic review demonstrated that an abnormal DWI MRI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 20.4 (95% CI 10.8 to 38.4) compared to those with normal MRI brain (Figure 32) [212, 325, 326, 329, 333, 338, 343, 346, 347, 349, 350, 353-355, 357, 361-364, 368, 370, 374, 375]. The meta-analysis included 1174 participants from 23 studies. Results were consistent across many studies, and abnormal MRI brain was associated with significantly increased OR of adverse outcome in 16 of 23 included studies. There was not conclusive evidence in the remaining 7 studies of a statistically significant relationship, and no study reported an association between abnormal MRI brain and decreased OR of adverse long-term outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  50.5, df 22). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was substantial ( $I^2$  56.5%, Tau 1.2). This likely reflects variation between studies on severity of NE in patients included, differences between studies regarding treatment with therapeutic hypothermia, duration of time to follow up, and variation in MRI technique and in particular the timing of MRI for which DWI results are very sensitive.



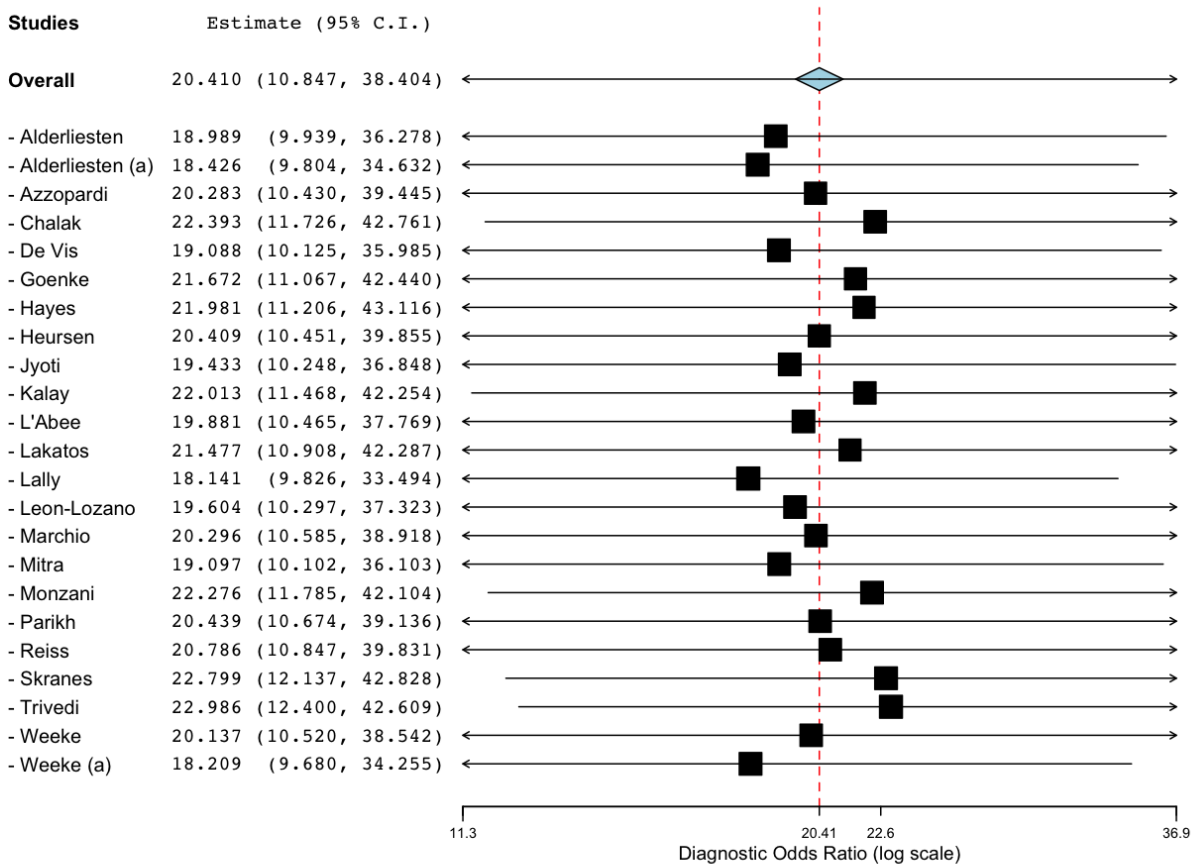
**Figure 32 Odds ratio of diffusion weighted MRI technique to predict long-term outcome in neonatal encephalopathy.** DWI result was associated with an increased odds ratio (OR) of long-term adverse outcome of 20.4 (95% CI 10.8 to 38.4) compared to those with normal MRI brain.

Diagnostic Random-Effects Model					
Metric: Diagnostic Odds Ratio					
Model Results					
Studies	Estimate	Lower bound	Upper bound	Std. error	p-Val
Overall	20.410	10.847	38.404	0.323	< 0.001
- Alderliesten	18.989	9.939	36.278	0.330	< 0.001
- Alderliesten (a)	18.426	9.804	34.632	0.322	< 0.001
- Azzopardi	20.283	10.430	39.445	0.339	< 0.001
- Chalak	22.393	11.726	42.761	0.330	< 0.001
- De Vis	19.088	10.125	35.985	0.324	< 0.001
- Goenke	21.672	11.067	42.440	0.343	< 0.001
- Hayes	21.981	11.206	43.116	0.344	< 0.001
- Heursen	20.409	10.451	39.855	0.341	< 0.001
- Jyoti	19.433	10.248	36.848	0.326	< 0.001
- Kalay	22.013	11.468	42.254	0.333	< 0.001
- L'Abée	19.881	10.465	37.769	0.327	< 0.001
- Lakatos	21.477	10.908	42.287	0.346	< 0.001
- Lally	18.141	9.826	33.494	0.313	< 0.001
- Leon-Lozano	19.604	10.297	37.323	0.329	< 0.001
- Marchio	20.296	10.585	38.918	0.332	< 0.001
- Mitra	19.097	10.102	36.103	0.325	< 0.001
- Monzani	22.276	11.785	42.104	0.325	< 0.001
- Parikh	20.439	10.674	39.136	0.331	< 0.001
- Reiss	20.786	10.847	39.831	0.332	< 0.001
- Skranes	22.799	12.137	42.828	0.322	< 0.001
- Trivedi	22.986	12.400	42.609	0.315	< 0.001
- Weeke	20.137	10.520	38.542	0.331	< 0.001
- Weeke (a)	18.209	9.680	34.255	0.322	< 0.001

**Table 8 Results of leave-one-out analysis for diffusion weighted imaging (DWI) studies to predict long-term outcome in neonatal encephalopathy.**



A sensitivity analysis was conducted using the leave-one-out method. Although some studies reported odds ratios that differed significantly from the composite OR, none were significantly different or carried sufficient weight to alter the composite OR independently (Figure 33) [212, 325, 326, 329, 333, 338, 343, 346, 347, 349, 350, 353-355, 357, 361-364, 368, 370, 374, 375]. See Table 9 [212, 325, 326, 329, 333, 338, 343, 346, 347, 349, 350, 353-355, 357, 361-364, 368, 370, 374, 375] for further details.

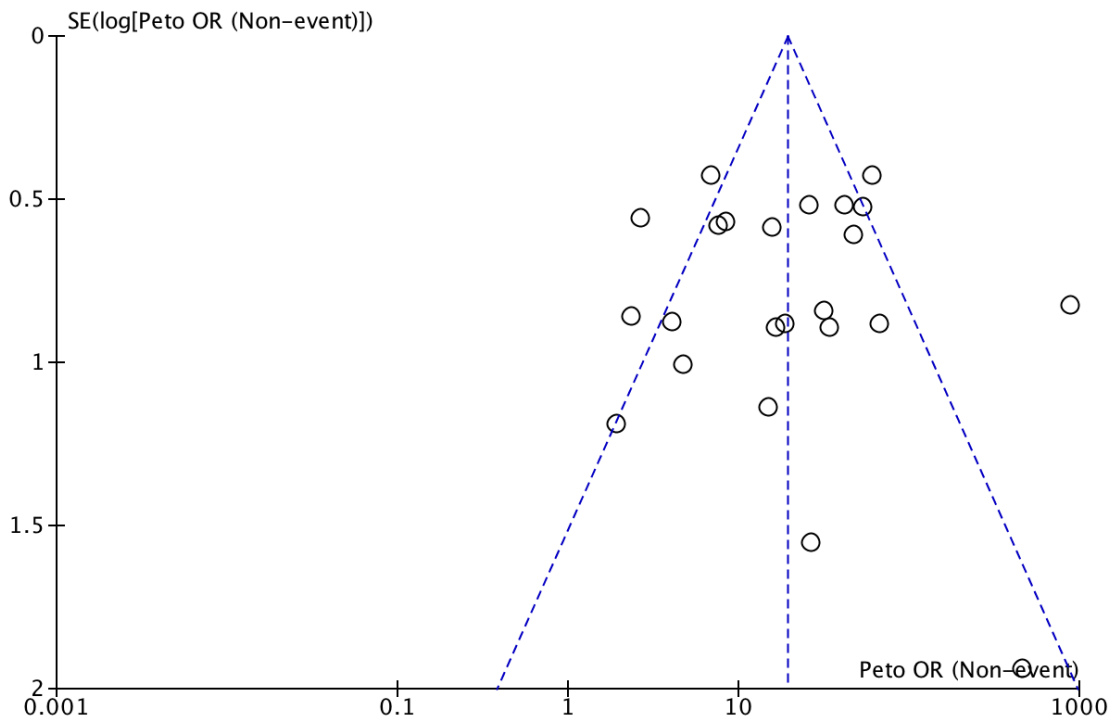


**Figure 33** Leave-one-out forest plot for studies including diffusion weighted imaging (DWI) results. No individual study had a significant effect alone on the overall results of the meta-analysis.

Diagnostic Random-Effects Model					
Metric: Diagnostic Odds Ratio					
Model Results					
Studies	Estimate	Lower bound	Upper bound	Std. error	p-Val
Overall	20.410	10.847	38.404	0.323	< 0.001
- Alderliesten	18.989	9.939	36.278	0.330	< 0.001
- Alderliesten (a)	18.426	9.804	34.632	0.322	< 0.001
- Azzopardi	20.283	10.430	39.445	0.339	< 0.001
- Chalak	22.393	11.726	42.761	0.330	< 0.001
- De Vis	19.088	10.125	35.985	0.324	< 0.001
- Goenke	21.672	11.067	42.440	0.343	< 0.001
- Hayes	21.981	11.206	43.116	0.344	< 0.001
- Heursen	20.409	10.451	39.855	0.341	< 0.001
- Jyoti	19.433	10.248	36.848	0.326	< 0.001
- Kalay	22.013	11.468	42.254	0.333	< 0.001
- L'Abée	19.881	10.465	37.769	0.327	< 0.001
- Lakatos	21.477	10.908	42.287	0.346	< 0.001
- Lally	18.141	9.826	33.494	0.313	< 0.001
- Leon-Lozano	19.604	10.297	37.323	0.329	< 0.001
- Marchio	20.296	10.585	38.918	0.332	< 0.001
- Mitra	19.097	10.102	36.103	0.325	< 0.001
- Monzani	22.276	11.785	42.104	0.325	< 0.001
- Parikh	20.439	10.674	39.136	0.331	< 0.001
- Reiss	20.786	10.847	39.831	0.332	< 0.001
- Skranes	22.799	12.137	42.828	0.322	< 0.001
- Trivedi	22.986	12.400	42.609	0.315	< 0.001
- Weeke	20.137	10.520	38.542	0.331	< 0.001
- Weeke (a)	18.209	9.680	34.255	0.322	< 0.001

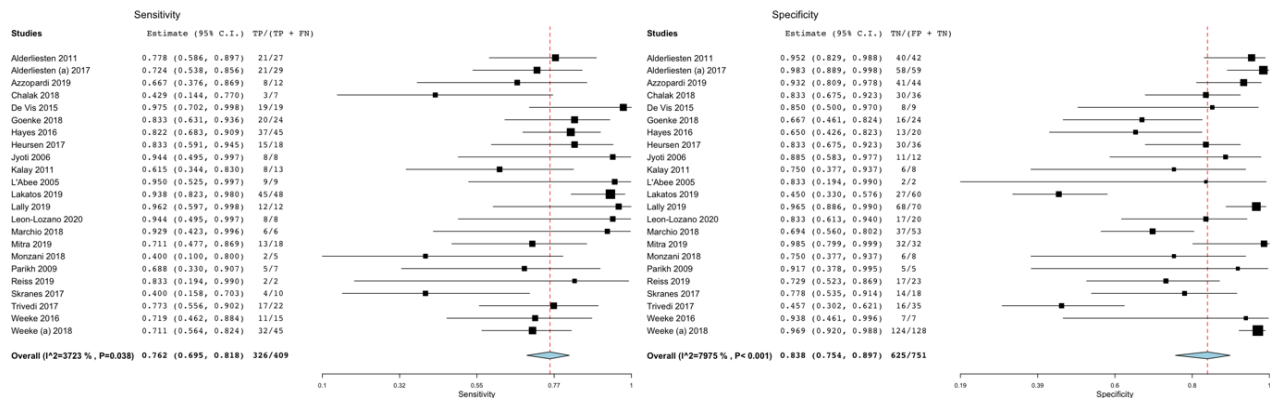
**Table 9 Results of leave-one-out analysis for diffusion weighted imaging (DWI) studies to predict long-term outcome in neonatal encephalopathy.**

Visual inspection of the funnel plot did not demonstrate significant asymmetry to suggest small study bias was a significant issue with this systematic review (Figure 34). The largest included studies are close to the summary estimate, and although some studies lie outside of the 95% confidence intervals, they are distributed on either side of the summary estimate line with a no predominance for small studies on either side of the summary effect line. Unfortunately, the neither the software OpenMetaAnalyst nor RevMan provide for statistical investigation of the effect of small study bias.



**Figure 34** Funnel plot of included studies for DWI to predict long-term outcome in neonatal encephalopathy. No significant issue with small study bias was evident.

Overall, MRI DWI technique was associated with a sensitivity of 76.2% (95% CI 70 to 81.8) to predict adverse long-term outcome ( $\text{Chi}^2$  35, df 22,  $I^2$  37.2%), and a specificity of 83.8% (95% CI 75.4 to 89.7) to predict adverse long-term outcome in NE ( $\text{Chi}^2$  108.7, df 22,  $I^2$  79.8%) (Figure 35) [212, 325, 326, 329, 333, 338, 343, 346, 347, 349, 350, 353-355, 357, 361-364, 368, 370, 374, 375]. This suggests that DWI on MRI brain is better at excluding those not at increased risk of adverse outcome and not as good at identifying those at increased risk. Both sensitivity and specificity results demonstrated significant heterogeneity, and this is likely in keeping with clinical variability discussed in previously meta-analyses above. In particular however, this may reflect the variability in timing of MRI, as DWI results pseudonormalise after day of life 6-7 in patients with neonatal encephalopathy.



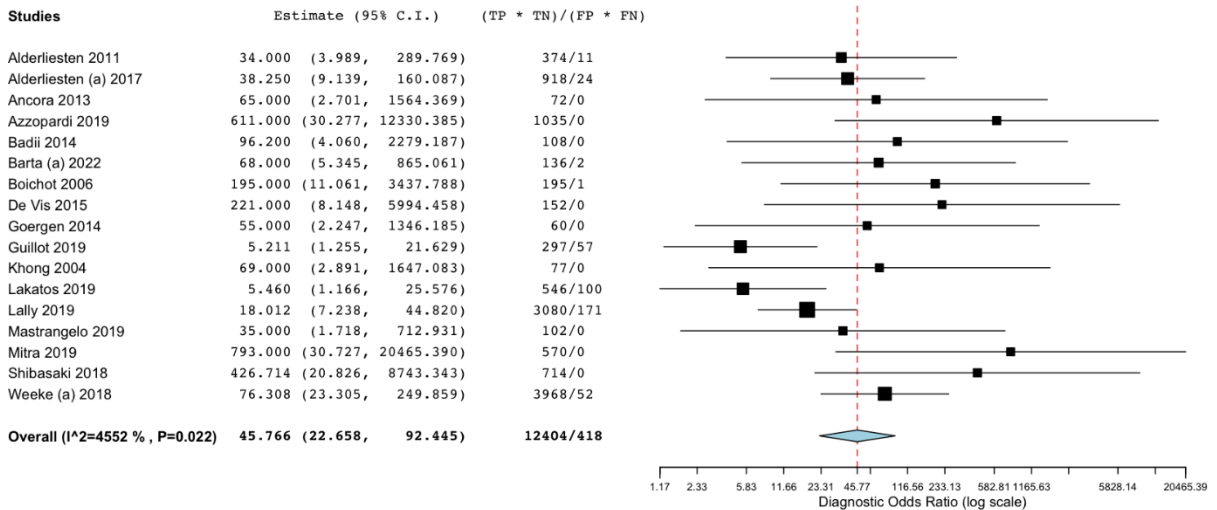
**Figure 35 Sensitivity and specificity of diffusion weighted MRI technique to predict long-term outcome in neonatal encephalopathy.** DWI technique was associated with a sensitivity of 76.2% (95% CI 70 to 81.8) to predict adverse long-term outcome ( $\text{Chi}^2$  35, df 22,  $I^2$  37.2%), and a specificity of 83.8% (95% CI 75.4 to 89.7) to predict adverse long-term outcome in NE ( $\text{Chi}^2$  108.7, df 22,  $I^2$  79.8%).

### MRI to predict outcome in NE, magnetic resonance spectroscopy only

Overall, the composite of studies of magnetic resonance spectroscopy (MRS) techniques included in this systematic review demonstrated that an abnormal MRS results was associated with an increased odds ratio (OR) of long-term adverse outcome of 45.8 (95% CI 22.7 to 92.4) compared to those with normal MRI brain (Figure 36) [104, 212, 310, 325-327, 329, 332-336, 338-376]. The meta-analysis included 1145 participants from 17 studies. Results were consistent across all studies, and abnormal MRI brain was associated with significantly increased OR of adverse outcome in all 17 included studies.

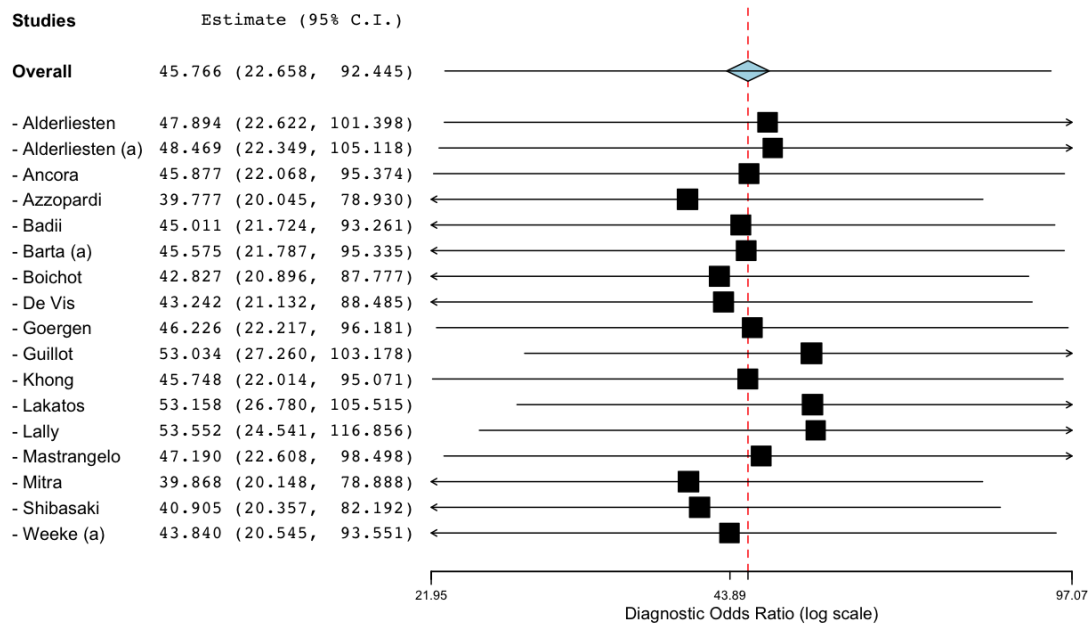
There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  29.4, df 16). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was moderate ( $I^2$  45.5%, Tau 0.8). This likely reflects clinical variation between studies on severity of NE in patients included, differences between studies

regarding treatment with therapeutic hypothermia, duration of time to follow up, and variation in MRS timing, metabolite measured, and anatomic area measured.



**Figure 36 Odds ratio of magnetic resonance spectroscopy technique to predict long-term outcome in neonatal encephalopathy.** An abnormal MRS results was associated with an increased odds ratio (OR) of long-term adverse outcome of 45.8 (95% CI 22.7 to 92.4) compared to those with normal MRI brain.

A sensitivity analysis was conducted using the leave-one-out method. Although some studies reported odds ratios that differed significantly from the composite OR, none were significantly different or carried sufficient weight to alter the composite OR independently (Figure 37) [104, 212, 310, 325-327, 329, 332-336, 338-376]. See Table 10 [104, 212, 310, 325-327, 329, 332-336, 338-376] for further details.

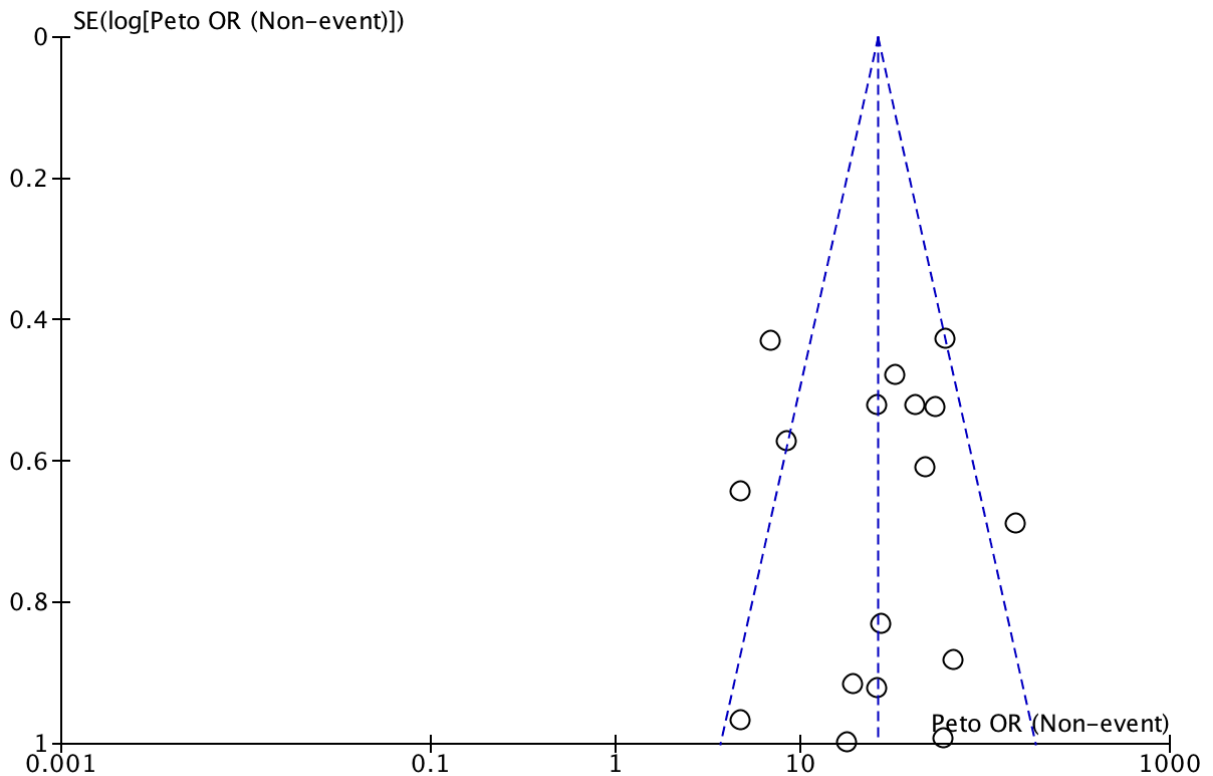


**Figure 37** Leave-one-out forest plot for studies including magnetic resonance spectroscopy results. No individual study had a significant effect alone on the overall results of the meta-analysis.

Diagnostic Random-Effects Model					
Metric: Diagnostic Odds Ratio					
Model Results					
Studies	Estimate	Lower bound	Upper bound	Std. error	p-Val
Overall	45.766	22.658	92.445	0.359	< 0.001
- Alderliesten	47.894	22.622	101.398	0.383	< 0.001
- Alderliesten (a)	48.469	22.349	105.118	0.395	< 0.001
- Ancora	45.877	22.068	95.374	0.373	< 0.001
- Azzopardi	39.777	20.045	78.930	0.350	< 0.001
- Badii	45.011	21.724	93.261	0.372	< 0.001
- Barta (a)	45.575	21.787	95.335	0.377	< 0.001
- Boichot	42.827	20.896	87.777	0.366	< 0.001
- De Vis	43.242	21.132	88.485	0.365	< 0.001
- Goergen	46.226	22.217	96.181	0.374	< 0.001
- Guillot	53.034	27.260	103.178	0.340	< 0.001
- Khong	45.748	22.014	95.071	0.373	< 0.001
- Lakatos	53.158	26.780	105.515	0.350	< 0.001
- Lally	53.552	24.541	116.856	0.398	< 0.001
- Mastrangelo	47.190	22.608	98.498	0.375	< 0.001
- Mitra	39.868	20.148	78.888	0.348	< 0.001
- Shibasaki	40.905	20.357	82.192	0.356	< 0.001
- Weeke (a)	43.840	20.545	93.551	0.387	< 0.001

**Table 10** Results of leave-one-out analysis for meta-analysis of magnetic resonance spectroscopy to predict long-term outcome in neonatal encephalopathy.

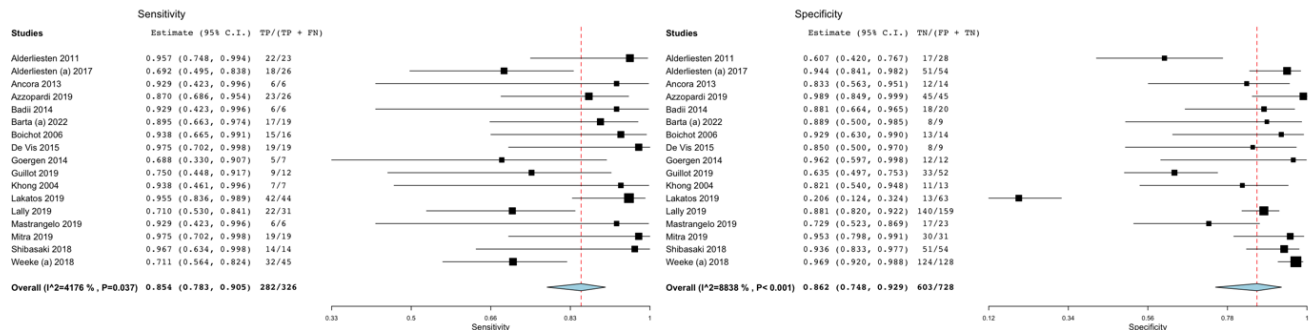
Visual inspection of the funnel plot did not demonstrate significant asymmetry to suggest small study bias was a significant issue with this systematic review (Figure 38). The largest included studies are close to the summary estimate, and although a very small number of studies lie outside of the 95% confidence intervals, they are distributed on either side of the summary estimate line with a no predominance for small studies on either side of the summary effect line.



**Figure 38** Funnel plot of included studies for magnetic resonance spectroscopy to predict long-term outcome in neonatal encephalopathy. No significant issue with small study bias was evident.

Overall, MRS technique was associated with a sensitivity of 85.4% (95% CI 78.3 to 90.5) to predict adverse long-term outcome (Chi<sup>2</sup> 27.5, df 16, I<sup>2</sup> 41.8%), and a specificity of 86.2% (95% CI 74.8 to 92.9) to predict adverse long-term outcome in NE (Chi<sup>2</sup> 137.7, df 16, I<sup>2</sup> 88.4%) (

Figure 39) [104, 212, 310, 325-327, 329, 332-336, 338-376]. This suggests that MRS brain is better at identifying those at higher risk of adverse outcome than to provide reassurance for those that are not at increased risk of adverse outcome. Both sensitivity and specificity results demonstrated significant heterogeneity, and this is likely in keeping with clinical variability discussed in previously meta-analyses above. However, this may reflect the variability in timing of MRS, the metabolites and ratios measured, and the anatomical regions examined.



**Figure 39 Sensitivity and specificity of magnetic resonance spectroscopy technique to predict long-term outcome in neonatal encephalopathy. MRS technique was associated with a sensitivity of 85.4% (95% CI 78.3 to 90.5) to predict adverse long-term outcome (Chi<sup>2</sup> 27.5, df 16, I<sup>2</sup> 41.8%), and a specificity of 86.2% (95% CI 74.8 to 92.9) to predict adverse long-term outcome in NE (Chi<sup>2</sup> 137.7, df 16, I<sup>2</sup> 88.4%).**



## Discussion

This is the largest systematic review and meta-analysis to date by included studies of MRI to predict long-term outcome in NE of which we are aware. 59 studies with 3092 participants were included in this review. Overall MRI was associated with a diagnostic odds ratio (DOR) of 23.7 (95% CI 16.7 to 33.5) increased risk of adverse outcome compared to those with normal MRI brain, and a sensitivity of 85% (95% CI 81.5 – 88%) and sensitivity of 76.4% (95% CI 71.3 – 80.8). The best MRI technique we identified in this study was magnetic resonance spectroscopy (MRS), giving a DOR of 45.8 (95% CI 22.7 – 92.4) compared to a DOR of 15.4 and 20.4 for conventional T1/T2 imaging and diffusion weighted imaging (DWI) respectively. Conventional T1/T2 imaging gave a very slightly superior sensitivity to predict adverse outcome of 85.6% (95% CI 81.5 – 88%) compared to MRS (85.4%) and DWI (76.2%). However, MRS gave a much superior specificity of 86.2% (95% CI 74.8 – 92.9%) compared to conventional T1/T2 imaging (69.5%) and DWI (83.9%). Although many sources of clinical and methodological variation between studies was identified, the total variability in effect estimates that is due to heterogeneity between studies was moderate in the total meta-analysis (42.1%), MRS meta-analysis (45.5%), and DWI meta-analysis (56%), and may not be significant in the T1/T2 meta-analysis (21.3%).

A recent large systematic review examined radiological and neurophysiological predictors of outcome in NE [211]. They examined the discriminatory ability of predictors including MRI, cranial USS, aEEG, EEG, near-infrared spectroscopy (NIRS), and

somatosensory evoked potentials (SEPs) to identify adverse outcome over 18 months of age. They included 22 studies of conventional T1/T2 imaging, and 13 studies of DWI, and examined the effect of timing of MRI and anatomical region studied on the DOR. They identified that the best predictors of outcome were MRI abnormalities in the posterior limb of the internal capsule (PLIC), abnormal ADC values in the thalamus, and MRS abnormalities in the basal ganglia & thalami, and aEEG abnormalities at 36 hours. It is not possible to compare the DOR, sensitivity, or specificity of each meta-analysis they completed with the results of our meta-analysis unfortunately, due to a lack of published data. Another systematic review of clinical tests to predict outcome in NE examined the value of MRI, aEEG, EEG, and SEPs [307]. This review limited results to those treated with TH and with neurological assessment at >18 months of age. They included 26 studies in total, 14 studies of MRI were included in a meta-analysis, and found that late MRI, at 7 days of age or older gave an AUC of 0.94 compared to 0.87 for total MRI, 0.88 for EEG, 0.78 for background aEEG, and 0.84 for SEP. The pooled sensitivity for total MRI was 85% and the pool specificity was 69%. The conclusion regarding timing of MRI was different in these two systematic reviews. Both studies identify the influence of MRI timing on prognostic value. The review by Ouwehand et al found that early MRI in the first week of life was a better predictor of neurodevelopmental outcome [211], and the study by Liu et al found that the later MRI,  $\geq 7$  days of life, provided slightly lower sensitivity but improved specificity and overall AUC compared to early MRI [307].

It is evident that each MRI technique has an optimal postnatal time to be completed. DWI changes are most sensitive for detecting abnormalities on MRI brain in the first few days of life [54], and pseudonormalise after 11-12 days in patients treated with TH which may underestimate the extent of brain injury [379]. Signal changes on conventional T1/T2 MRI are most evolve over the first 2 weeks of life and are most evident from 10 days of life [317], although some studies have suggested early or late timing does not influence the predictive ability of conventional imaging [315]. The predictive ability of MRS metabolite changes are excellent within the first 2 weeks of life, but appears to best when completed between days 7 – 14 [380]. Therefore, both timing and technique will interact to influence the prognostic value of MRI in NE. In this study we focused on the influence of MRI technique on the prognostic value of MRI, regardless of timing, however it would be of additional benefit to examine the prognostic value of each technique within its optimal time window. One complication is that many studies reported MRI completion within a wide time window (Table 5), therefore making it difficult to match MRI technique to timing of MRI in each study. A further complication is which anatomical regions and functional connections to focus on in the interpretation of the prognostic value of the MRI [381]. 47% of asymptomatic newborns who underwent MRI had incidental findings that were not associated with significant differences in neurodevelopmental outcome [316]. Therefore it is important to finding the anatomical patterns associated with adverse prognosis in NE, and whether these are influenced by technique and timing of MRI brain [210]. Although not identified a priori as a question to be answered by this systematic review (Prospero - CRD42021286461), the data extracted for this would be sufficient to provide an answer to

these questions. Another consideration is the predictive ability of scoring systems to identify those at risk of neurodevelopmental disability, and whether they provide superior results to other reporting mechanisms [382].

Some limitations of this study are the lack of inclusion of timing of MRI assessment on the interpretation of predictive value of MRI brain. As outlined above timing has a significant influence on the predictive value of different MRI techniques, and combination of both MRI technique and timing would provide the best discriminatory ability for MRI in NE. A further limitation is the limited overlap of methodology for studies of MRS. Although as a technique this provided the best predictive value, each study included different metabolites and anatomic regions in which these were measured, with very little overlap between studies. This may explain the wide confidence intervals for these results, and replication of some of the included studies it would be of great value. We included studies with earlier developmental assessment compared to other recent systematic reviews. This may result in an underestimate of the predictive value of MRI as some neurodevelopmental impairments may not be evident until later, however we judged that inclusion of greater number of studies would increase the certainty of the results for this systematic review.

## Conclusion

It is evident that MRI provides excellent prognostic value for infants with NE, and to date provides superior discriminatory ability compared to EEG, aEEG, NIRS, and serum or other biochemical markers. It is also increasingly clear that for improved outcomes, infants

with NE at risk of neurodevelopmental disability require as early identification as possible [213], especially as increasing numbers of interventions in the very early postnatal period become more available [214]. The facilities available for MRI and clinical considerations regarding the condition of the infant will influence the timing and technique of MRI used for infants with NE, but this study should provide a basis for optimum choice of technique and improved interpretation of results when MRI is performed for these infants.

## Chapter 4: Biomarkers to predict outcome in neonatal encephalopathy: a systematic review and meta-analyses

### Abstract

**Introduction:** Neonatal encephalopathy (NE) biomarkers are important for early diagnosis, guiding prognosis, improved understanding of the disease pathophysiology, and identification of potential therapeutic agents. Many biomarkers have been investigated in recent years as potential prognostic markers, however none are used in routine clinical practice. We examined current evidence of serum biomarkers to predict short- and long-term outcome in neonatal encephalopathy.

**Methods:** The review was prospectively registered with PROSPERO. EMBASE, PubMed, World of Science and the Cochrane Library databases were searched for studies that reported prognostic serum biomarkers in participants with NE. Participants were dichotomised by groups into normal or adverse outcome, defined as death or abnormal neurodevelopmental outcome at >12 months of age. 2 reviewers independently completed the screening and data extraction. Risk of bias was completed using QUIPS tool. The mean difference in serum biomarkers between groups was calculated using a random effects model.

**Results :** Literature search provided 3046 results. 98 studies of over 20 different biomarkers were eligible for qualitative synthesis, however only 41 studies could be included due to variation in biomarker measurement and reporting. Meta-analyses for serum interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-10, NSE, S100, S100B, and TNF- $\alpha$  to predict adverse outcome were completed. Serum IL-6, IL-8, NSE, and TNF- $\alpha$  and were all lower in participants with normal long-term outcome. Serum IL-6 and lactate were lower in participants with normal short-term outcome. However, there was substantial variability in results between included studies.

**Discussion:** Several biomarkers provide promising prognostic value in NE. Lower IL-6, NSE, and TNF- $\alpha$  were associated with normal long-term outcome in patients with NE. This relationship requires further evaluation. Many studies could not be included a MA and greater uniformity in biomarker outcome reporting would allow improved evidence synthesis.

## Introduction

Neonatal Encephalopathy (NE) describes central nervous system dysfunction in the first few days of life in term and near-term infants [1]. It has a multifactorial aetiology [2]. The incidence of NE is between 0.5-3 per 1000 live births in high-income countries but is much more frequent in low-income settings [12]. NE is associated with high incidence of early mortality and disability in survivors [204]. NE is difficult to diagnose, to treat, and to predict outcome. Currently diagnosis and prognosis are based on clinical, radiological and electrophysiological markers when available [211]. However, accurate prediction of prognosis and identification of patients at risk of adverse neurodevelopmental outcome remains a significant challenge [213]. In recent years there has been extensive research in blood, urine and cerebrospinal fluid (CSF) biomarkers in NE [383]. These biomarkers may help identify infants at high risk of NE, objectively classify severity of NE, predict response to treatment and prognosticate outcome. Despite this research, there is no gold standard biomarker known at present. Although prediction of adverse outcomes is difficult, there is increasing evidence for the benefit of early recognition and intervention for high patients [214]. With limited resources it is essential to identify those patients at high risk so that they can be prioritised for early intervention services. Identifying prognostic biomarkers has implications for counselling parents on expected neurodevelopmental outcomes, future adjunctive therapies, and guiding research.



## Aims

To assess the current evidence for biomarkers to predict outcome in neonatal encephalopathy.

## Hypothesis

Serum and cerebrospinal biomarkers would provide long- and short-term prognostic information on the outcome for patients with neonatal encephalopathy.

## Methods

The study was prospectively registered with Prospero (CRD42017056763). The systematic review followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and the Cochrane Handbook.

### Search Strategy

Four major databases, EMBASE, MEDLINE, World of Science and the Cochrane Library were systematically searched in January 2017 for studies published between January 1966 and December 2016. A repeat search, using an identical search strategy was conducted in March 2021, as the original search was out of date. The search strategy included the following relevant terms: 'neonatal' or 'newborn', 'neonatal encephalopathy' or 'hypoxic ischaemic encephalopathy' or 'perinatal asphyxia', 'biomarker' or 'biological indicator' or 'serum marker'. The complete search strategy and MESH terms used are reported in Appendix VI, Table 34. Authors were contacted for additional details not included in the publication if they were required for inclusion in a meta-analysis.

### Eligibility Criteria

Studies were eligible for inclusion if they reported prognostic biomarkers for NE in patients  $\geq 35$  weeks gestational age and neurodevelopmental outcomes at ages  $\geq 12$  months. Studies that used any definition of NE, HIE or birth asphyxia which included features of encephalopathy in the first 24 hours of life were included in the qualitative analysis. To be included in a meta-analysis studies had to report outcomes that could be categorised as

'normal' or 'adverse'. Studies also had to report continuous data for prognostic biomarkers in means and standard deviations, or data that could be converted reliably into means and standard deviations to be included in a meta-analysis. Studies that included patients <35 weeks gestational age at birth were excluded. Studies that included patients who received TH and those that did not were included in the study. Case reports were excluded but studies of all other design types were included. No language or publication restrictions were applied.

#### Outcome Definition

The primary outcome was defined as death or adverse neurodevelopmental outcome at  $\geq 12$  months of age. Neurodevelopmental outcomes used to define adverse outcome included non-survival, cerebral palsy, developmental delay, abnormal neurological exam, and several standardised tests of child development including Bayley Scales of Infant Development and Griffith Scales. The timing, reported outcome, and definition of adverse outcome for each included study is outlined in the Appendix.

#### Study Search and Selection

Results from the study search were uploaded to Covidence software and duplicates were removed. The title, abstract and full text screening were conducted independently by 2 investigators (MOD and TH for the original search, and TH, AB, PS, and ST for the updated search). Disagreements were resolved by consensus. Studies deemed eligible for inclusion

were categorised by prognostic biomarker and included in the meta-analysis if outcomes were reported in a comparable manner.

#### Bias & Quality Assessment

Risk of bias for included studies was assessed using the Quality in Prognostic Studies (QUIPS) tool. Each study was assessed and assigned a rating of low, moderate, or high risk of bias according to 6 criteria: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding and statistical analysis and reporting. Assessment was completed independently by 2 researchers (MOD & TH) and disagreements resolved by consensus. Studies with  $\geq 1$  criteria at high risk of bias were excluded from the meta-analysis.

#### Data Extraction

A standard data extraction table was created following the CHARMS (Checklist for critical Appraisal and data extraction for systematic Reviews of prediction Modelling Studies) checklist. The following data was collected: characteristics of included studies, patient characteristics, prognostic factors and outcome definitions and measurements. The data extraction form was completed independently by 2 researchers (MOD and TH) and disagreements were resolved by consensus. To assimilate studies using different statistics for reported outcomes, conversion was completed when required using the methods described by Hozo et al, Wan et al, and Luo et al [384-386]. When missing data was encountered, authors were contacted by email to request data.

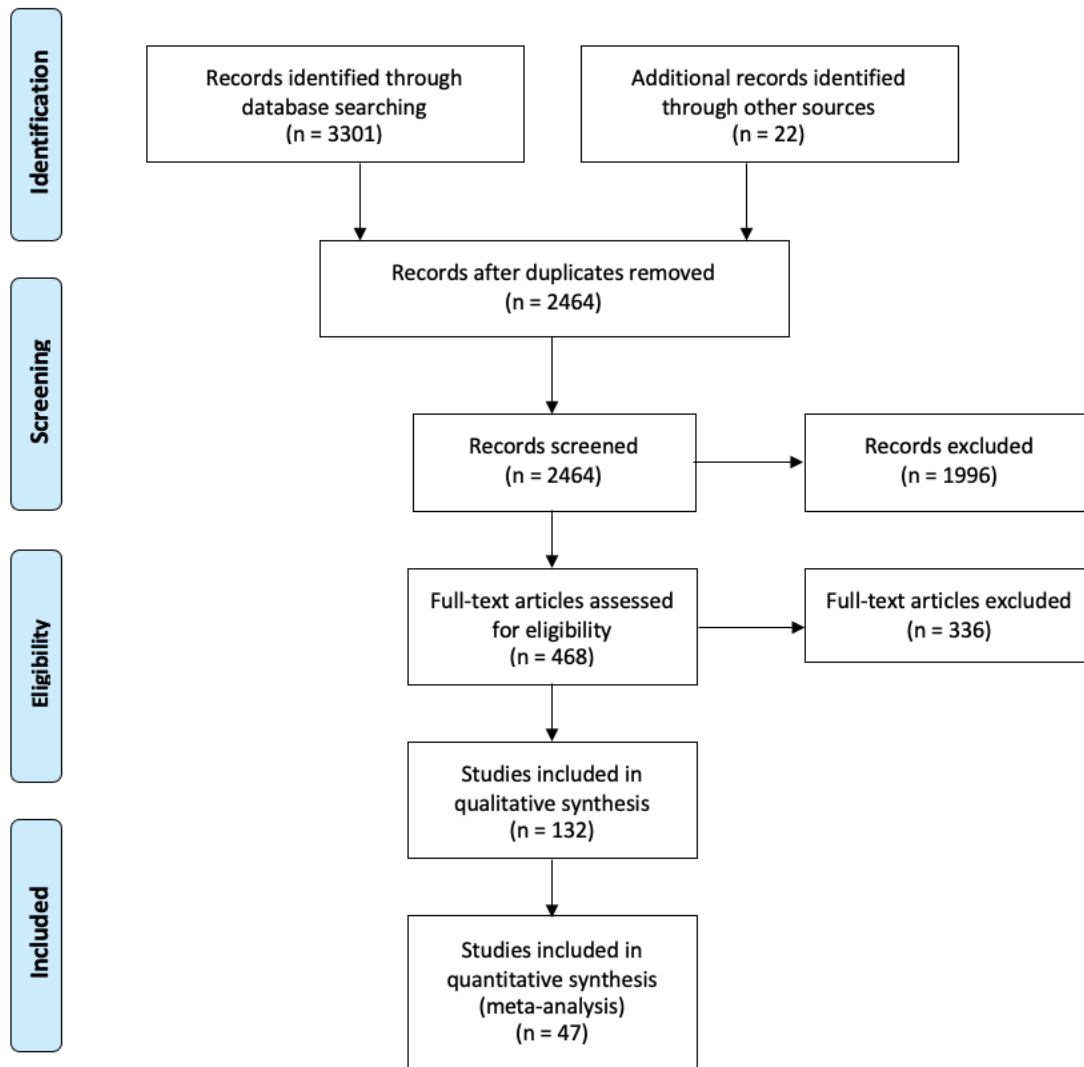
## Statistical Analysis

Meta-analysis and statistical analysis were completed using RevMan software (v5.3), the Cochrane networks software for writing systematic reviews and conducting meta-analyses. A composite adverse outcome of death and abnormal neurodevelopmental outcome was used. For categorical data the odds ratio (OR) of adverse outcome was calculated with 95% confidence intervals. Between group mean differences and 95% confidence intervals were calculated for each biomarker for which a meta-analysis was completed, to compare normal and abnormal outcome groups. A random effects models was used due to differences in patient demographic profiles between studies, differences in severity profiles between studies, and differences in the use of TH between studies. The leave-one-out method was used to assess the effect of individual studies on the pooled effect estimate. Small study effects were assessed by visual inspection of the funnel plot. We intended to quantify the effect using the Peters linear regression test, however no meta-analysis included more than 10 studies, and in keeping with Cochrane guidelines (10.4.3.1) no tests for funnel plot asymmetry were conducted. Statistical tests were completed using RevMan (v5.3) software. Tests were 2-tailed, and results were considered significant at the level  $p < .05$ .

## Results

### Literature search and screening results (study selection)

2464 studies were identified by the literature search following removal of duplicates (Figure 40). Grey literature sources provided a further 22 studies. Following title and abstract screening there were 468 studies suitable for full text screening. 132 studies were included for qualitative synthesis. From these 132 studies, over 30 individual prognostic biomarkers were identified. 47 studies could be included in meta-analyses for biomarkers to predict long- or short-term outcomes in NE.



**Figure 40 PRISMA flow diagram demonstrating the study selection process for this systematic review.**

### Characteristics of included studies

Articles were published between 1994 and 2021. They originated from more than 20 different countries. The 47 studies included in the meta-analyses included over 1000 participants, ranging from 19 to 95 participants per study. There were over 50 different primary outcomes reported from studies included in the meta-analyses. Studies of all design types including cohort, retrospective and prospective, case-control studies, and randomised controlled trials if the non-intervention group could be examined independently were included in this systematic review. All studies were conducted in a hospital setting.

### Risk of bias in included studies

The risk of bias for each included study was assessed using the QUIPS tool. Each study was assessed by study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. A study was judged to be overall high risk of bias if one or more domains were judged to be at high risk of bias. We intended to exclude any study that was judged to be high risk of bias, however this was not required for the current review.

### Serum biomarkers to predict long-term outcome in neonatal encephalopathy

Long term outcome was defined as mortality or abnormal neurodevelopmental assessment at  $\geq 12$  months of age. Definition of abnormal neurodevelopmental assessment included any clinical definition of abnormal neurodevelopment or formal assessment using a validated scale such as Bayley-III or Griffiths assessment. The number of eligible studies



for analysis of individual serum & CSF biomarkers ranged from 2 to 8, however the highest number of studies included in any meta-analysis was reduced and limited due to variability in predictor measurement and reporting methods. None of the included studies had  $\geq 2$  criteria for high risk of bias. A meta-analysis was completed if there were at least 2 studies which provided data that could be synthesised appropriately. We completed meta-analyses for 7 serum biomarkers to predict long-term outcome in NE (Table 14) and for 3 cerebrospinal fluid biomarkers to predict long-term outcome in NE (Table 15). For all completed meta-analyses we compared continuous data for the biomarker from 2 groups, a normal or good outcome group compared to the adverse outcome group. The mean difference and 95% confidence interval between groups was then measured using a random effects model.

Meta-analyses could be completed for 7 serum prognostic biomarkers to predict long-term outcome in NE. Although only one study was included in the forest plot for S100B, it is included for comparison with S100. In total 1077 individual participant results are included in the study, from 13 different studies. Included studies were published between 1998 and 2021, and included participants treated and not treated with TH. From the meta-analyses here was evidence to suggest a difference in IL-6, IL-8, NSE, and TNF- $\alpha$  between groups. The only included study of S100B also suggested a difference between groups, but there was no evidence from this study for S100.

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
2.1 IL-1beta	4	142	Mean Difference (IV, Random, 95% CI)	-0.60 [-1.52, 0.32]
2.2 IL-6	6	260	Mean Difference (IV, Random, 95% CI)	-70.22 [-139.89, -0.55]
2.3 IL-8	3	103	Mean Difference (IV, Random, 95% CI)	-15.72 [-27.85, -3.59]
2.4 IL-10	2	115	Mean Difference (IV, Random, 95% CI)	-24.15 [-52.47, 4.17]
2.5 NSE	3	129	Mean Difference (IV, Random, 95% CI)	-40.93 [-63.70, -18.17]
2.6 S100	3	120	Mean Difference (IV, Random, 95% CI)	3.16 [-8.92, 15.24]
2.7 S100B	1	24	Mean Difference (IV, Random, 95% CI)	-22.62 [-36.26, -8.98]
2.8 TNF-alpha	5	184	Mean Difference (IV, Random, 95% CI)	-20.84 [-35.45, -6.24]

**Table 14 Summary of serum biomarkers to predict long-term outcome in neonatal encephalopathy.**

### *Serum interleukin-6*

Higher serum IL-6 was associated with increased risk of adverse outcome (Figure 41) [100, 102, 107, 168, 240, 387]. The meta-analysis included 260 participants from six studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a higher mean IL-6 of 70.22 pg/ml (95% CI 0.55 – 139.89) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher IL-6 with abnormal outcome, although only two of six studies reached statistical significance, and there is substantial overlap of confidence intervals. However, the size of the effect varied significantly between studies and the point estimates are highly disparate. Mean IL-6 for those with normal outcome varied between 17 pg/ml and 69 pg/ml, and varied between 73.2 pg/ml and 258815 pg/ml for those with abnormal outcome. Due to very high SD, some of the larger studies by participant inclusion were given very small weighting in the meta-analysis. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  10.48, df 5). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was substantial ( $I^2$  52%, Tau 2740). One included study has extremely wide confidence intervals. Further analysis of this study did not indicate any risk of bias and exclusion of the study did not affect the conclusions of the meta-analysis [102]. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum interleukin-8*

Higher serum IL-8 was associated with increased risk of adverse outcome (Figure 42) [102, 107, 168]. The meta-analysis included 103 participants from three studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a higher mean IL-8 of 15.72 pg/ml (95% CI 3.59 – 27.85) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher IL-8 with abnormal outcome, although only one study reached statistical significance, and there is substantial overlap of confidence intervals. However, the size of the effect varied significantly between studies and the point estimates are highly disparate. Mean IL-8 for those with normal outcome varied between 40.7 pg/ml and 154 pg/ml, and varied between 55.8 pg/ml and 2535 pg/ml for those with abnormal outcome. Due to very high SD in two of the three included studies, one study is given very substantial weighting in the meta-analysis and negligible weighting to the remaining two studies. This explains the lack of statistical heterogeneity in the study ( $\text{Chi}^2$  1.15 df 2) and why the proportion of total variability in the meta-analysis attributable to between-study heterogeneity was very small ( $I^2$  0%, Tau 0.00). These tests do not accurately reflect the heterogeneity in the meta-analysis nor the between study variability which is evident from visual inspection of the forest plot. One included study has extremely wide confidence intervals. Further analysis of this study did not indicate any risk of bias and exclusion of the study did not affect the conclusions of the meta-analysis. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum NSE*

Higher serum NSE was associated with increased risk of adverse outcome (Figure 43) [388-390]. The meta-analysis included 124 participants from three studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a higher mean NSE of 38.95 pg/ml (95% CI 18.33 – 59.57) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher NSE with abnormal outcome, two of the three studies reached statistical significance, and there is moderate overlap of confidence intervals. However, the size of the effect varied significantly between studies and the point estimates are moderately disparate. Mean NSE for those with normal outcome varied between 9.9 pg/ml and 53.76 pg/ml, and varied between 72.9 pg/ml and 76.9 pg/ml for those with abnormal outcome. All included studies were given significant weighting in the meta-analysis. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  4.25, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was substantial ( $I^2$  53%, Tau 176). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum TNF- $\alpha$*

Higher serum TNF- $\alpha$  was associated with increased risk of adverse outcome (Figure 44) [168, 240, 387, 391, 392]. The meta-analysis included 184 participants from five studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a

higher mean TNF- $\alpha$  of 20.84 pg/ml (95% CI 6.24 – 35.45) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher NSE with abnormal outcome, although only two of the five studies reached statistical significance, and there is substantial overlap of confidence intervals for four of the five included studies. However, the size of the effect varied significantly between studies and the point estimates are moderately disparate. Mean TNF- $\alpha$  for those with normal outcome varied between 4.55 pg/ml and 155.2 pg/ml, and varied between 6.79 pg/ml and 252 pg/ml for those with abnormal outcome. All included studies were given significant weighting in the meta-analysis. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 61.12, df 4). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable (I<sup>2</sup> 93%, Tau 217). One study reported substantially higher mean TNF- $\alpha$  for both groups and the study was an outlier compared to the other four included studies. Further analysis of this study did not indicate any risk of bias and exclusion of the study did not affect the conclusions of the meta-analysis, although it did substantially affect the effect size. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum S100*

There was no evidence in this meta-analysis of a difference in serum S100 between participants with normal or abnormal outcome in NE (Figure 45) [240, 393]. The meta-analysis included 93 participants from two studies. Both included studies were at moderate

risk of bias. The point estimate suggested that serum S100 was 9.88 pg/ml higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 22.53 pg/ml to -2.76 pg/ml. There was no consistency in effect direction between studies and both confidence intervals crossed the line of no effect. The confidence interval is very wide for one study, which explains why there is substantial overlap between both confidence intervals. The point estimates are highly disparate, however. No individual study reported a significant association between measured S100 and outcome. Mean S100 for those with normal outcome varied between 32.9 pg/ml and 56 pg/ml, and varied between 17 pg/ml and 24.3 pg/ml for those with abnormal outcome. One of the included studies was given significant weighting in the meta-analysis, and one was weighted very small due to a very large SD. There is heterogeneity in the meta-analysis as evidenced by the wide variation in confidence intervals ( $\text{Chi}^2$  0.9, df 1). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was statistically low ( $I^2$  0%), however only two studies are included, and we judge that this does not represent the clinical heterogeneity between studies. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum S100B*

There was no evidence in this meta-analysis of a difference in serum S100B between participants with normal or abnormal outcome in NE (Figure 46) [389, 394]. The meta-analysis included 51 participants from two studies. Both included studies were at moderate

risk of bias. The point estimate suggested that serum S100B was 12.04 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 7.05 pg/ml to -31.12 pg/ml. There was no consistency in effect direction between studies. One study reported a significant association between measured S100B and outcome however the confidence interval for one study crossed the line of no effect. There is very limited overlap of the confidence intervals from included studies and the point estimates are highly disparate. Mean S100B for those with normal outcome varied between 4.18 pg/ml and 4.74 pg/ml, and varied between 7.82 pg/ml and 26.8 pg/ml for those with abnormal outcome. Both included studies were given significant weighting in the meta-analysis. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  5.86, df 1). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was substantial ( $I^2$  83%). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum interleukin-1 $\beta$*

There was no evidence in this meta-analysis of a difference in serum IL-1 $\beta$  between participants with normal or abnormal outcome in NE (Figure 47) [168, 240, 387, 391]. The meta-analysis included 142 participants from four studies. All included studies were at moderate risk of bias. The point estimate suggested that IL-1 $\beta$  was 0.6 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.52 pg/ml to 0.32 pg/ml. All 4 included studies found IL-1 $\beta$  was lower in

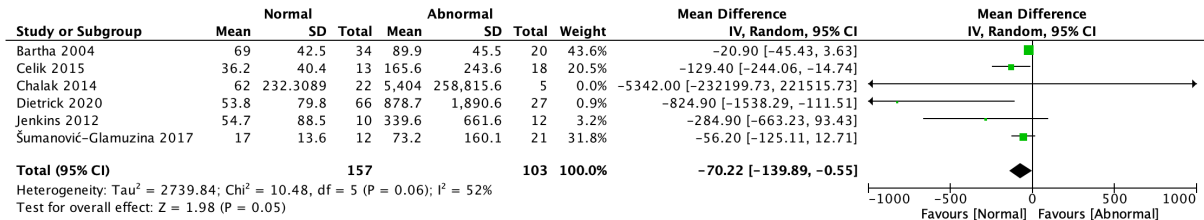


those with normal outcomes however this finding was only significant in two of the four included studies. There is significant overlap of confidence intervals and the point estimates are close for three of the four included studies, however the point estimates are all very close to the line of no effect. One included study has a much wider confidence interval which does not overlap with other included studies and its point effect is highly disparate from the other included studies. The weighting of this study in the meta-analysis is very low. Further analysis of this study did not indicate any significant risk of bias and exclusion of the study did not affect the conclusions of the meta-analysis. Mean IL-1 $\beta$  for those with normal outcome varied between 0.18 pg/ml and 65 pg/ml, and varied between 0.7 pg/ml and 86.3 pg/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 14.23, df 3). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable (I<sup>2</sup> 79%, Tau 0.49). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

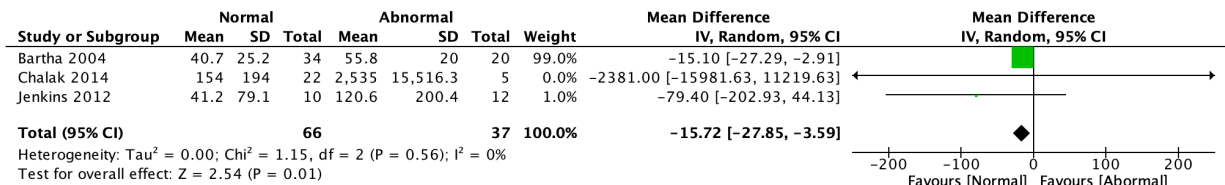
### *Serum interleukin-10*

There was no evidence in this meta-analysis of a difference in serum IL-10 between participants with normal or abnormal outcome in NE (Figure 48) [100, 107]. The meta-analysis included 115 participants from two studies. All included studies were at moderate risk of bias. The point estimate suggested that IL-10 was 24.15 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI

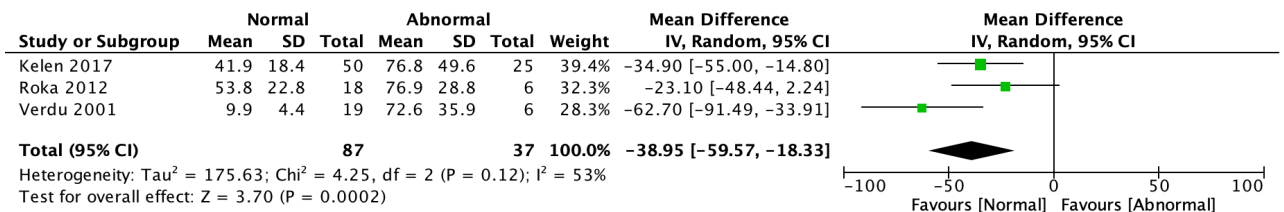
ranged from 52.47 pg/ml to -4.17 pg/ml. Both included studies found IL-1 $\beta$  was lower in those with normal outcomes however this finding was not statistically significant in either study. There is significant overlap of confidence intervals, and the point estimates are close for both included studies. Mean IL-10 for those with normal outcome varied between 20 pg/ml and 25.4 pg/ml, and varied between 44.5 pg/ml and 55.2 pg/ml for those with abnormal outcome. There is low heterogeneity in the meta-analysis but ( $\text{Chi}^2$  0.27, df 1). This requires cautious interpretation as there are only two studies included in the meta-analysis. The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was low ( $I^2$  0%, Tau 0.00). We are interpreting the finding of no significant difference in IL-10 between groups with caution and as providing very low-level evidence only. We suggest that the question requires further investigation.



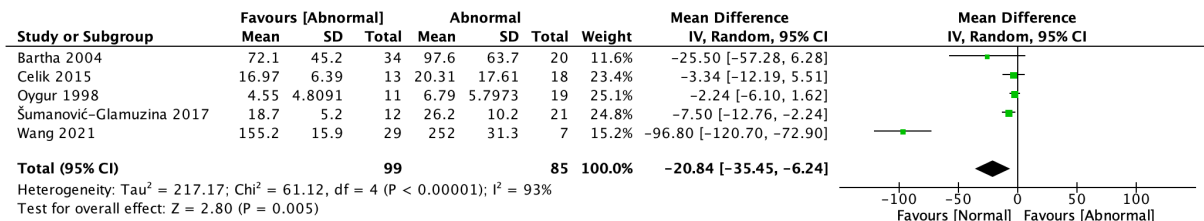
**Figure 41** Forest plot comparison of serum IL-6 between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean IL-6 of 70.22 pg/ml (95% CI 0.55 – 139.89) compared to patients who survived with normal neurodevelopmental outcome.



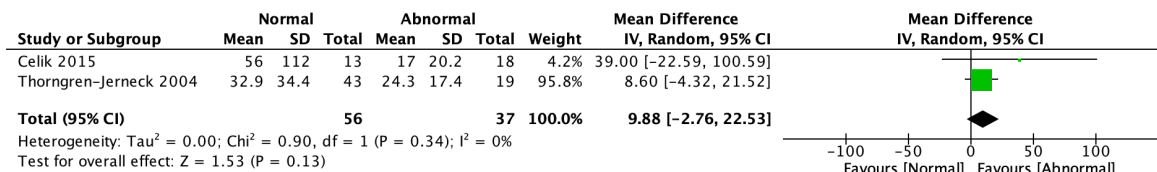
**Figure 42** Forest plot comparison of serum IL-8 between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean IL-8 of 15.72 pg/ml (95% CI 3.59 – 27.85) compared to patients who survived with normal neurodevelopmental outcome.



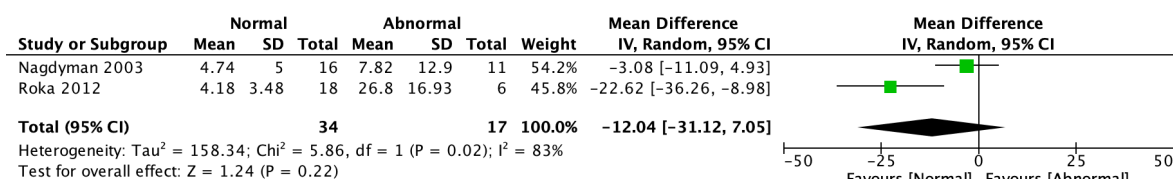
**Figure 43** Forest plot comparison of serum NSE between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean NSE of 38.95 pg/ml (95% CI 18.33 – 59.57) compared to patients who survived with normal neurodevelopmental outcome.



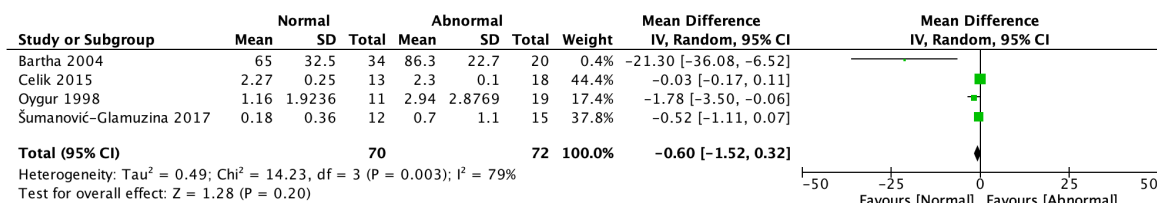
**Figure 44** Forest plot comparison of serum TNF-α between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean TNF-α of 20.84 pg/ml (95% CI 6.24 – 35.45) compared to patients who survived with normal neurodevelopmental outcome.



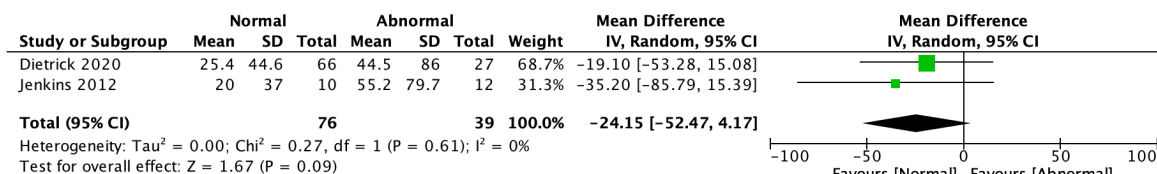
**Figure 45 Forest plot comparison of serum S100 between groups with normal and abnormal outcome in NE.** There was no significant difference in serum S100 between groups. The point estimate suggested that serum S100 was 9.88 pg/ml higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 22.53 pg/ml to -2.76 pg/ml.



**Figure 46 Forest plot comparison of serum S100B between groups with normal and abnormal outcome in NE.** There was no significant difference in serum S100B between groups. The point estimate suggested that serum S100B was 12.04 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 7.05 pg/ml to -31.12 pg/ml.



**Figure 47 Forest plot comparison of serum IL-1β between groups with normal and abnormal outcome in NE.** There was no significant difference in serum IL-1β between groups. The point estimate suggested that IL-1β was 0.6 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.52 pg/ml to 0.32 pg/ml.



**Figure 48 Forest plot comparison of serum IL-10 between groups with normal and abnormal outcome in NE.** There was no significant difference in serum IL-10 between groups. The point estimate suggested that IL-10 was 24.15 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 52.47 pg/ml to -4.17 pg/ml.

Cerebrospinal fluid biomarkers to predict long-term outcome in neonatal encephalopathy

A meta-analysis could be completed for only 3 cerebrospinal fluid prognostic biomarkers to predict long-term outcome in NE. Although only one study was included in the forest plot for both IL-6 and S100, they were included for comparison with serum biomarker results. In total 390 individual participant results are included in the study, from 8 different studies. Included studies were published between 1998 and 2020, and included participants treated and not treated with TH. From the meta-analyses here was no evidence to suggest a difference in any of the potential CSF biomarkers.

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
3.1 IL-1beta	3	85	Mean Difference (IV, Random, 95% CI)	-4.52 [-12.36, 3.31]
3.2 IL-6	1	33	Mean Difference (IV, Random, 95% CI)	-25.02 [-47.32, -2.72]
3.3 NSE	5	175	Mean Difference (IV, Random, 95% CI)	-1.85 [-4.73, 1.02]
3.4 S100	1	23	Mean Difference (IV, Random, 95% CI)	-0.39 [-0.77, -0.01]
3.5 TNF-alpha	3	74	Mean Difference (IV, Random, 95% CI)	-1.72 [-10.78, 7.33]

*Table 15 Summary of cerebrospinal fluid biomarkers to predict long-term outcome in neonatal encephalopathy.*

### *Cerebrospinal fluid IL-1 $\beta$*

There was no evidence in this meta-analysis of a difference in CSF IL-1 $\beta$  between participants with normal or abnormal outcome in NE (Figure 49) [118, 387, 391]. The meta-analysis included 85 participants from three studies. All included studies were at moderate risk of bias. The point estimate suggested that IL-1 $\beta$  was 4.52 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -3.31 ng/ml to 12.36 ng/ml. All three included studies found IL-1 $\beta$  was lower in those with normal outcomes however this finding was only significant in one of the three included studies. The measured level of CSF IL-1 $\beta$  in the study by Sumanovic-Glamuzina was so low in both groups that it was not possible to calculate a measurement of effect and it did not provide any data for the meta-analysis. There is significant overlap of confidence intervals, although the confidence interval for one study is very wide, and the point estimates are disparate for the two relevant included studies. Mean CSF IL-1 $\beta$  for those with normal outcome varied between 0.00 ng/ml and 42.4 ng/ml and varied between 0.014 ng/ml and 58.74 ng/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 1.25, df 1). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was low (I<sup>2</sup> 20%, Tau 17.41), but this is likely to be an underestimate of due to the small number of included studies and the high weighting of one of the included studies. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Cerebrospinal fluid NSE*

There was no evidence in this meta-analysis of a difference in CSF NSE between participants with normal or abnormal outcome in NE (Figure 50) [275, 355, 395-397]. The meta-analysis included 175 participants from five studies. All included studies were at moderate risk of bias. The point estimate suggested that NSE was 1.85 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.02 ng/ml to 4.73 ng/ml. All five included studies found NSE was lower in those with normal outcomes and this finding was significant in four of the five included studies. One of the studies given significant weighting in the meta-analysis was the outlier study that did not find a significant association between CSF NSE and outcome, and this explains why the meta-analysis did not find a significant relationship despite this consistency. Although the direction of effect is consistent across all included studies, there is minimal overlap of confidence intervals, and the point estimates are disparate for the three of the included studies. Mean CSF NSE for those with normal outcome varied between 0.62 ng/ml and 29.1 ng/ml, and varied between 1.01 ng/ml and 213.3 ng/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  55.54, df 4). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  93%, Tau 4.34). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without. A further study by Celtik et al which was not included in the meta-analysis due to insufficient



reported details, demonstrated good discriminatory predictor of poor outcome with cut-off of 45 ng/mL, with a sensitivity of 84% and specificity of 70% [398].

### *Cerebrospinal fluid TNF- $\alpha$*

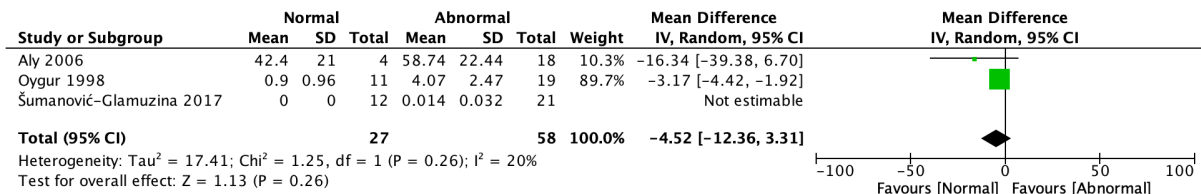
There was no evidence in this meta-analysis of a difference in CSF TNF- $\alpha$  between participants with normal or abnormal outcome in NE (Figure 51) [118, 387, 391]. The meta-analysis included 74 participants from three studies. All included studies were at moderate risk of bias. The point estimate suggested that TNF- $\alpha$  was 1.72 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -7.33 ng/ml to 10.78 ng/ml. None of the included studies found a significant difference in CSF TNF- $\alpha$  between groups. There is significant overlap of confidence intervals, although the confidence interval for two studies are wide, and the point estimates are moderately disparate. Mean CSF TNF- $\alpha$  for those with normal outcome varied between 17.6 ng/ml and 74.2 ng/ml, and varied between 19.2 ng/ml and 116.63 ng/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 2.68, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was low (I<sup>2</sup> 25%, Tau 24.42), but this is likely to be an underestimate of due to the small number of included studies and the high weighting of one of the included studies. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Cerebrospinal fluid IL-6*

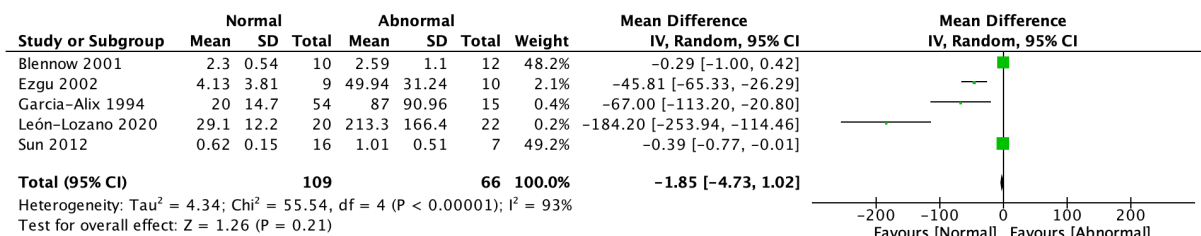
Continuous data could only be extracted for one study and it is presented in a forest plot. Sumanovic-Glamuzina et al included 33 participants in their study and reported lower CSF IL-6 in participants with normal outcome following NE compared to those with abnormal outcome (Figure 52) [387]. Participants with normal outcome had mean CSF IL-6 25.02 pg/ml lower (95% CI 2.72 – 47.32) than those with abnormal outcome. Two other studies reported an association between CSF IL-6 and outcome in NE but did not report sufficient detail to be included in the meta-analysis. Dietrick et al. reported significant and negative correlations between CSF IL-6 and Bayley-III motor ( $p = 0.02$ ) and Bayley-III cognitive outcomes ( $p = 0.05$ ) [100]. Martin-Ancel reported higher CSF IL-6 levels in participants with adverse outcome compared to those with favourable outcome [399].

### *Cerebrospinal fluid S100*

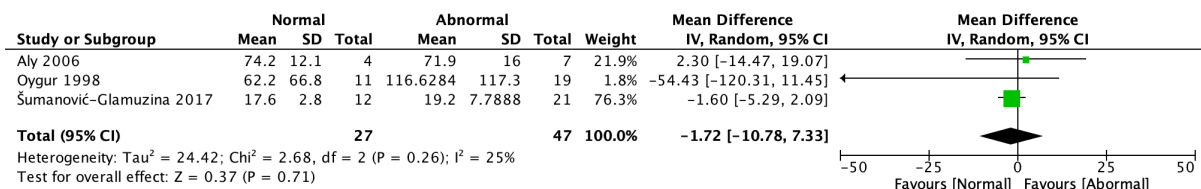
Continuous data could only be extracted for one study and it is presented in a forest plot. Sun et al included 23 participants in their study and reported lower CSF S100 in participants with normal outcome following NE compared to those with abnormal outcome (Figure 53) [275]. Participants with normal outcome had mean CSF S100 0.39 ng/ml lower (95% CI 0.01 – 0.77) than those with abnormal outcome. No interpretation can be made beyond the only included study, and this requires further exploration and validation in other studies.



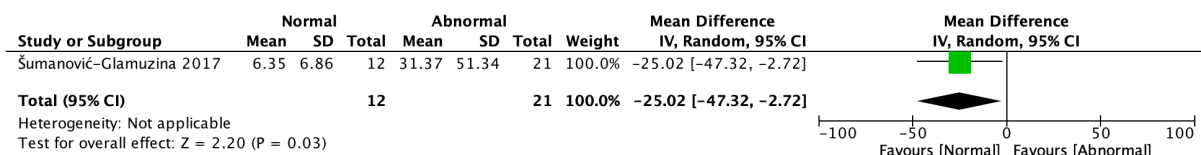
**Figure 49 Forest plot comparison of cerebrospinal fluid(interleukin) IL-1β between groups with normal and abnormal outcome in NE.** There was no significant difference in cerebrospinal fluid IL-1β between groups. The point estimate suggested that IL-1β was 4.52 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -3.31 ng/ml to 12.36 ng/ml.



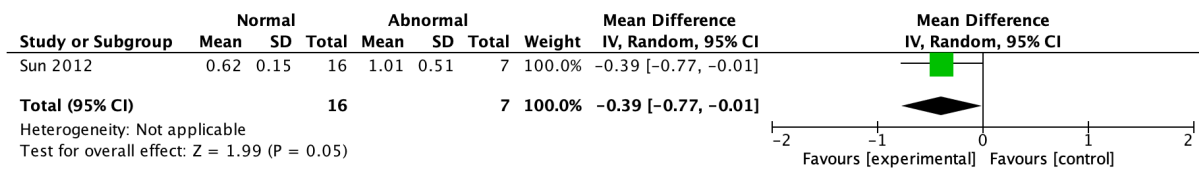
**Figure 50 Forest plot comparison of cerebrospinal fluid NSE between groups with normal and abnormal outcome in NE.** There was no significant difference in cerebrospinal fluid NSE between groups. The point estimate suggested that NSE was 1.85 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.02 ng/ml to 4.73 ng/ml.



**Figure 51 Forest plot comparison of cerebrospinal fluid TNF-α between groups with normal and abnormal outcome in NE.** There was no significant difference in cerebrospinal fluid TNF-α between groups. The point estimate suggested that TNF-α was 1.72 ng/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -7.33 ng/ml to 10.78 ng/ml.



**Figure 52 Forest plot comparison of cerebrospinal fluid IL-6 between groups with normal and abnormal outcome in NE.** Only one study is included in the forest plot so no meta-analysis is possible.



**Figure 53 Forest plot comparison of cerebrospinal fluid S100 between groups with normal and abnormal outcome in NE. Only one study is included in the forest plot so no meta-analysis is possible.**

## Serum and blood biomarkers to predict short-term outcomes

Long term outcome was defined as mortality in the first 28 days of life, abnormal MRI brain or higher severity on clinical assessment within the first week of life. The number of eligible studies for analysis of individual serum & CSF biomarkers ranged from 2 to 12, however the number of studies included in any meta-analysis was reduced and limited due to variability in predictor measurement and reporting methods. None of the included studies had  $\geq 2$  criteria for high risk of bias. A meta-analysis was completed if there were at least 2 studies which provided data that could be synthesised appropriately. We completed meta-analyses for 11 serum biomarkers to predict short-term outcome in NE (Table 16). For all completed meta-analyses we compared continuous data for the biomarker from 2 groups, a normal or good outcome group compared to the adverse outcome group. The mean difference and 95% confidence interval between groups was then measured using a random effects model.

Meta-analyses could be completed for 11 serum prognostic biomarkers to predict short-term outcome in NE. In total 1742 individual participant results are included in the study, from 26 different studies. Included studies were published between 1998 and 2017, and included participants treated and not treated with TH. From the meta-analyses there was evidence to suggest a difference in serum IL-6 and lactate between groups.

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
1.1 Glucose	4	149	Mean Difference (IV, Random, 95% CI)	0.05 [-0.84, 0.95]
1.2 Ionized calcium	3	83	Mean Difference (IV, Random, 95% CI)	0.05 [-0.08, 0.18]
1.3 IL-1beta	3	74	Mean Difference (IV, Random, 95% CI)	-7.72 [-28.75, 13.30]
1.4 IL-6	5	172	Mean Difference (IV, Random, 95% CI)	-22.57 [-44.49, -0.66]
1.5 Lactate	4	267	Mean Difference (IV, Random, 95% CI)	-4.50 [-6.19, -2.81]
1.6 Lactate Dehydrogenase	5	290	Mean Difference (IV, Random, 95% CI)	-947.66 [-2071.96, 176.65]
1.7 Nucleated red blood cells	2	149	Mean Difference (IV, Random, 95% CI)	-13.15 [-35.86, 9.56]
1.8 S100	3	105	Mean Difference (IV, Random, 95% CI)	-10.65 [-22.78, 1.48]
1.9 TNF-alpha	5	170	Mean Difference (IV, Random, 95% CI)	-14.27 [-42.60, 14.05]
1.10 Troponin	3	191	Mean Difference (IV, Random, 95% CI)	-1.29 [-2.84, 0.26]
1.11 White Cell Count	2	122	Mean Difference (IV, Random, 95% CI)	2.04 [-0.91, 5.00]

*Table 16 Summary of serum and blood biomarkers to predict short-term outcome in neonatal encephalopathy.*

### *Serum IL-6*

Higher serum IL-6 was associated with increased risk of adverse outcome (Figure 54) [102, 240, 400-402]. The meta-analysis included 172 participants from five studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a higher mean IL-6 of 22.57 pg/ml (95% CI 0.66 – 44.49) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher IL-6 with abnormal outcome, although only two of five studies reached statistical significance. There is substantial overlap of confidence intervals, although the confidence intervals for some studies are quite wide, and point estimates for each individual study are all close to the summary point estimate. Mean IL-6 for those with normal outcome varied between 2.67 pg/ml and 118.96 pg/ml, and varied between 23.31 pg/ml and 1650.8 pg/ml for those with abnormal outcome. Due to very high SD, some of the included studies were given very small weighting in the meta-analysis. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  6.2, df 4). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was moderate ( $I^2$  35%, Tau 198.7). One included study has extremely wide confidence intervals [102]. Further analysis of this study did not indicate any risk of bias and exclusion of the study did not affect the conclusions of the meta-analysis. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Blood lactate*

Higher serum lactate was associated with increased risk of adverse outcome (Figure 55) [403-406]. The meta-analysis included 267 participants from four studies. All included studies were at moderate risk of bias. Adverse outcome was associated with a higher mean lactate of 4.5 mg/dL (95% CI 0.66 – 44.49) compared to patients who survived with normal neurodevelopmental outcome. Individual study results consistently associated higher lactate with abnormal outcome, with three of four studies reached statistical significance. There is substantial overlap of confidence intervals, although the confidence intervals for some studies are quite wide. Point estimates for each individual study are all close to the summary point estimate. Mean lactate for those with normal outcome varied between 7.1 mg/dL and 13.1 mg/dL, and varied between 11.09 mg/dL and 23.48 mg/dL for those with abnormal outcome. Due to very high SD, some of the included studies were given very small weighting in the meta-analysis. There is evidence of heterogeneity in the meta-analysis although the statistical tests do not confirm this ( $\text{Chi}^2$  2.88, df 3). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was very small ( $I^2$  0%, Tau 0.00), but this is likely to be an underestimate due to very small weighting given to two of the studies with large standard deviations. Further analysis of these studies did not indicate any risk of bias and exclusion of these studies did not affect the conclusions of the meta-analysis. Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.



### *Blood glucose*

There was no evidence in this meta-analysis of a difference in serum glucose between participants with normal or abnormal outcome in NE (Figure 56) [407-410]. The meta-analysis included 149 participants from four studies. All included studies were at moderate risk of bias. The point estimate suggested that serum glucose was 0.05 mmol/L higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.84 mmol/L to 0.95 mmol/L. Only one of the included studies found a significant difference in serum glucose between groups. There is significant overlap of confidence intervals, although the confidence interval for two studies are wide, and the point estimates are widely disparate and are on both sides of the line of no effect. Mean serum glucose for those with normal outcome varied between 2.8 mmol/L and 5.8 mmol/L, and varied between 1.7 mmol/L and 8.3 mmol/L for those with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  75.19, df 3). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  96%, Tau 0.6). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum ionised calcium*

There was no evidence in this meta-analysis of a difference in serum ionised calcium between participants with normal or abnormal outcome in NE (Figure 57) [409, 411, 412]. The meta-analysis included 83 participants from three studies. All included studies were at

moderate risk of bias. The point estimate suggested that serum ionised calcium was 0.05 mmol/L higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.08 mmol/L to 0.18 mmol/L. Only one of the included studies found a significant difference in serum glucose between groups. There is significant overlap of confidence intervals, although the confidence interval for one study is very wide, and the point estimates are widely disparate and are on both sides of the line of no effect. Mean serum ionised calcium for those with normal outcome varied between 1.12 mmol/L and 1.47 mmol/L, and varied between 0.99 mmol/L and 1.57 mmol/L for those with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  20.10, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  90%, Tau 0.01). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum IL-1 $\beta$*

There was no evidence in this meta-analysis of a difference in serum IL-1 $\beta$  between participants with normal or abnormal outcome in NE (Figure 58) [118, 240, 391]. The meta-analysis included 74 participants from three studies. All included studies were at moderate risk of bias. The point estimate suggested that serum IL-1 $\beta$  was 7.72 ng/mL lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 13.31 ng/mL to -28.75 ng/mL. Two of the included studies found a significant difference in serum glucose between groups, however the measurement of effects were in

opposite directions. There is no significant overlap of confidence intervals, and the confidence interval for two studies are very wide. The point estimates are widely disparate and are on both sides of the line of no effect. Mean serum IL-1 $\beta$  for those with normal outcome varied between 5.68 ng/ml and 42.4 ng/ml, and varied between 2.36 ng/ml and 75.5 ng/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 10.04, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable (I<sup>2</sup> 80%, Tau 268.9). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

#### *Serum lactate dehydrogenase*

There was no evidence in this meta-analysis of a difference in serum lactate dehydrogenase (LDH) between participants with normal or abnormal outcome in NE (Figure 59) [403, 411, 413-415]. The meta-analysis included 290 participants from five studies. All included studies were at moderate risk of bias. The point estimate suggested that serum LDH was 947.66 IU/L lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -2071.96 IU/L to 176.65 IU/L. Two of the included studies found a significant difference in serum LDH between groups, and both found LDH was lower in participants with normal outcome. There is no significant overlap of confidence intervals, and the confidence interval for all studies are very wide. The point estimates are widely disparate and are on both sides of the line of no effect. Mean serum IL-

1 $\beta$  for those with normal outcome varied between 701 IU/L and 2304.55 IU/L, and varied between 1014 IU/L and 2830.75 IU/L for those with abnormal outcome. There is significant heterogeneity in the meta-analysis (Chi<sup>2</sup> 33.32, df 4). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable (I<sup>2</sup> 88%, Tau 1162726.32). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

#### *Blood nucleated red blood cells*

There was no conclusive evidence in this meta-analysis of a difference in serum nucleated red blood cells (nRBC) between participants with normal or abnormal outcome in NE (Figure 60) [416, 417]. The meta-analysis included 149 participants from two studies. Both included studies were at moderate risk of bias. The point estimate suggested that serum nRBC was 13.15 nRBC/mm<sup>4</sup> lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -9.56 nRBC/mm<sup>4</sup> to 35.86 nRBC/mm<sup>4</sup>. Both of the included studies found a significant difference in serum nRBC between groups, and both found nRBC was lower in participants with normal outcome. There is no overlap of confidence intervals, and the confidence interval for both studies are very wide which explains the lack of a measurable difference in the meta-analysis despite both included studies finding a significant difference. The point estimates are widely disparate. Mean serum nRBC for those with normal outcome varied between 0.7 nRBC/mm<sup>4</sup> and 2.02 nRBC/mm<sup>4</sup>, and varied between 3.81 nRBC/mm<sup>4</sup> and 25.67 nRBC/mm<sup>4</sup> for those

with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  45.96, df 1). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  98%, Tau 262.81). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum S100*

There was no evidence in this meta-analysis of a difference in serum S100 between participants with normal or abnormal outcome in NE (Figure 61) [240, 393, 394]. The meta-analysis included 105 participants from three studies. All included studies were at moderate risk of bias. The point estimate suggested that serum S100 was 10.65 ng/mL lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.48 ng/mL to 22.78 ng/mL. None of the included studies found a significant difference in serum S100 between groups. There is significant overlap of confidence intervals for all included studies. The point estimates are moderately disparate. Mean serum S100 for those with normal outcome varied between 3.15 ng/mL and 32.83 ng/mL, and varied between 18.25 ng/mL and 35.53 ng/mL for those with abnormal outcome. There is evidence of heterogeneity in the meta-analysis although the statistical tests do not confirm this ( $\text{Chi}^2$  1.68, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was small ( $I^2$  0%, Tau 0.00). Despite the statistical tests, some sources of clinical heterogeneity include variation in timing of

sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

### *Serum TNF- $\alpha$*

There was no evidence in this meta-analysis of a difference in serum TNF- $\alpha$  between participants with normal or abnormal outcome in NE (Figure 62) [102, 240, 391, 400, 401]. The meta-analysis included 170 participants from five studies. All included studies were at moderate risk of bias. The point estimate suggested that serum TNF- $\alpha$  was 14.27 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -14.05 pg/ml to 42.6 pg/ml. Only one of the included studies found a significant difference in serum TNF- $\alpha$  between groups, and found TNF- $\alpha$  was lower in participants with normal outcome. There is some overlap of confidence intervals for four of the five studies, however one confidence interval is extremely wide and one study is an outlier. The point estimates are widely disparate for two of the studies, and consistent for three of the studies. Mean serum TNF- $\alpha$  for those with normal outcome varied between 4.4 pg/ml and 44.32 pg/ml, and varied between 6.27 pg/ml and 90.23 pg/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  460.94, df 4). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  99%, Tau 852.97). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

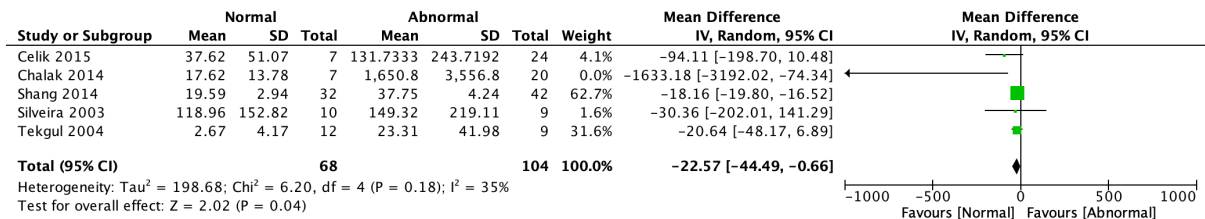
### *Serum troponin*

There was no conclusive evidence in this meta-analysis of a difference in serum troponin between participants with normal or abnormal outcome in NE (Figure 63) [406, 418, 419]. The meta-analysis included 191 participants from three studies. All included studies were at moderate risk of bias. The point estimate suggested that serum troponin was 1.28 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.27 pg/ml to 2.84 pg/ml. All three of the included studies found a significant difference in serum troponin between groups, and all found troponin was lower in participants with normal outcome. However, while there is substantial overlap of confidence interval for two of the studies, there is one outlier study [406]. The confidence intervals for two of the studies are very wide which explains the lack of a measurable difference in the meta-analysis despite all included studies finding a significant difference. The point estimates are widely disparate. Mean serum troponin for those with normal outcome varied between 0.02 pg/ml and 0.08 pg/ml and varied between 0.08 pg/ml and 2.24 pg/ml for those with abnormal outcome. There is significant heterogeneity in the meta-analysis ( $\text{Chi}^2$  29.62, df 2). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was considerable ( $I^2$  93%, Tau 1.73). Some sources of this heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

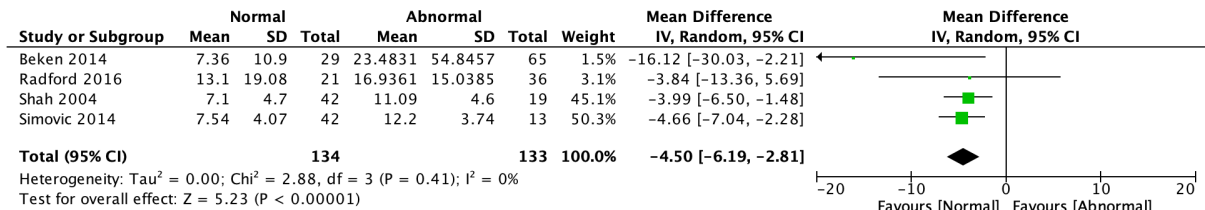
### *Blood white cell count*

There was no conclusive evidence in this meta-analysis of a difference in serum white cell count (WCC) between participants with normal or abnormal outcome in NE (Figure 64) [403, 411]. The meta-analysis included 122 participants from two studies. Both included studies were at moderate risk of bias. The point estimate suggested that serum WCC was 2.04 WCC/mm<sup>3</sup> higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.91 WCC/mm<sup>3</sup> to 5.00 WCC/mm<sup>3</sup>. Neither of the included studies found a significant difference in serum WCC between groups. There is substantial overlap of confidence interval for the two included studies, however the confidence intervals for the two studies are very wide and the point estimates are moderately disparate. Mean serum WCC for those with normal outcome varied between 23.7 WCC/mm<sup>3</sup> and 25.9 WCC/mm<sup>3</sup>, and varied between 22.3 WCC/mm<sup>3</sup> and 22.7 WCC/mm<sup>3</sup> for those with abnormal outcome. There is evidence of heterogeneity in the meta-analysis although the statistical tests do not confirm this (Chi<sup>2</sup> 0.72, df 1). The proportion of total variability in the meta-analysis attributable to between-study heterogeneity was small (I<sup>2</sup> 0%, Tau 0.00). Despite the statistical tests, some sources of clinical heterogeneity include variation in timing of sampling, method of biomarker measurement, and the inclusion of participants undergoing therapeutic hypothermia and those without.

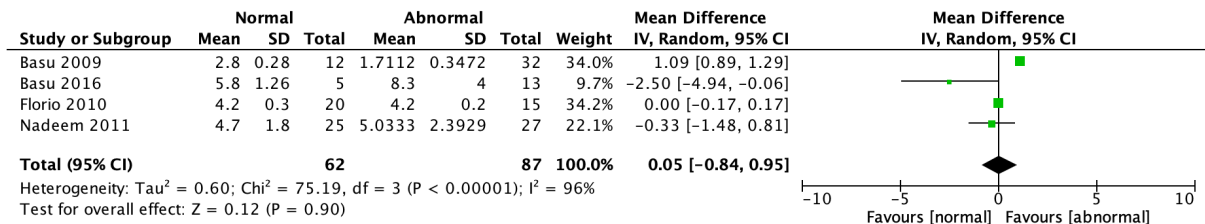




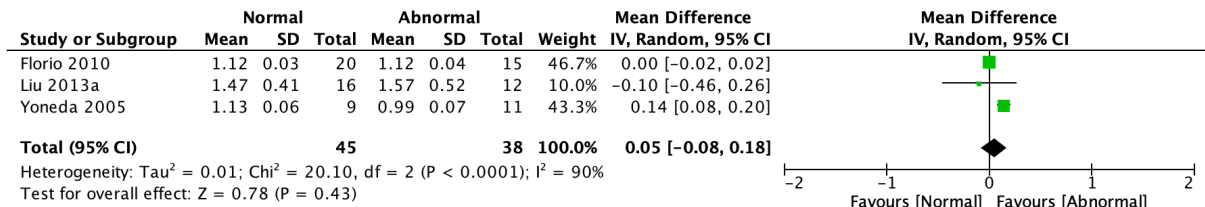
**Figure 54 Forest plot comparison of serum IL-6 between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean IL-6 of 22.57 pg/ml (95% CI 0.66 – 44.49) compared to patients who survived with normal neurodevelopmental outcome.**



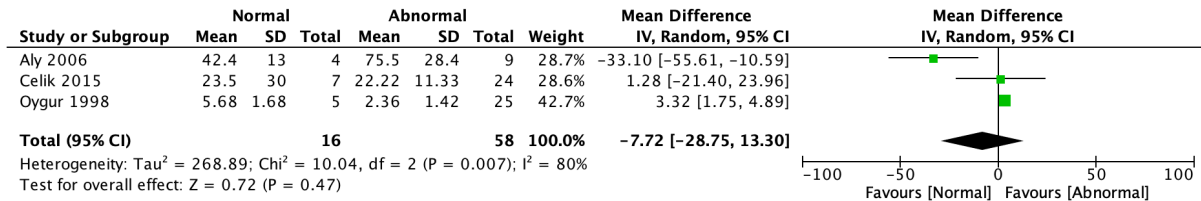
**Figure 55 Forest plot comparison of serum lactate between groups with normal and abnormal outcome in NE. Adverse outcome was associated with a higher mean lactate of 4.5 mg/dL (95% CI 0.66 – 44.49) compared to patients who survived with normal neurodevelopmental outcome.**



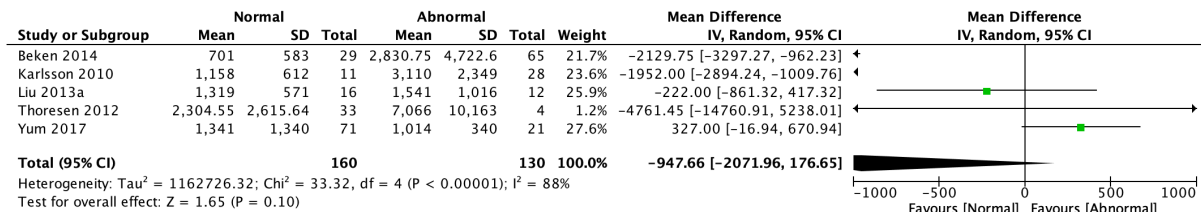
**Figure 56 Forest plot comparison of serum glucose between groups with normal and abnormal outcome in NE. There was no significant difference in serum glucose between groups. The point estimate suggested that serum glucose was 0.05 mmol/L higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.84 mmol/L to 0.95 mmol/L.**



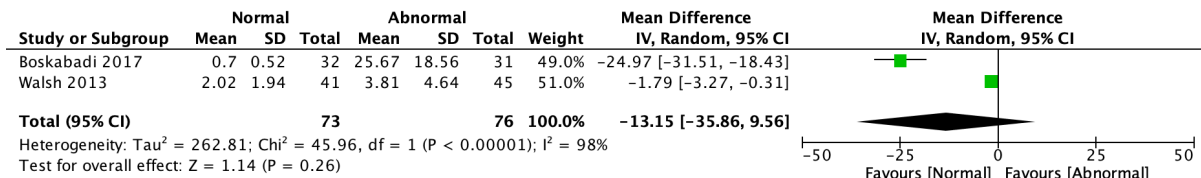
**Figure 57 Forest plot comparison of serum ionised calcium between groups with normal and abnormal outcome in NE. There was no significant difference in serum ionised calcium between groups. The point estimate suggested that serum ionised calcium was 0.05 mmol/L higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.08 mmol/L to 0.18 mmol/L.**



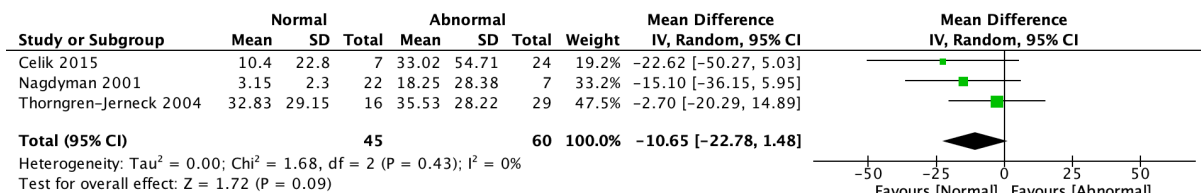
**Figure 58 Forest plot comparison of serum IL-1β between groups with normal and abnormal outcome in NE.** There was no significant difference in serum IL-1β between groups. The point estimate suggested that serum IL-1β was 7.72 ng/mL lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from 13.31 ng/mL to -28.75 ng/mL.



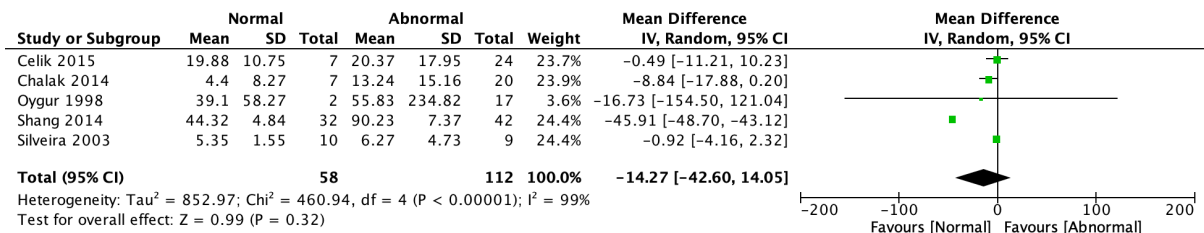
**Figure 59 Forest plot comparison of serum lactate dehydrogenase between groups with normal and abnormal outcome in NE.** There was no significant difference in serum lactate dehydrogenase between groups. The point estimate suggested that serum LDH was 947.66 IU/L lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -2071.96 IU/L to 176.65 IU/L.



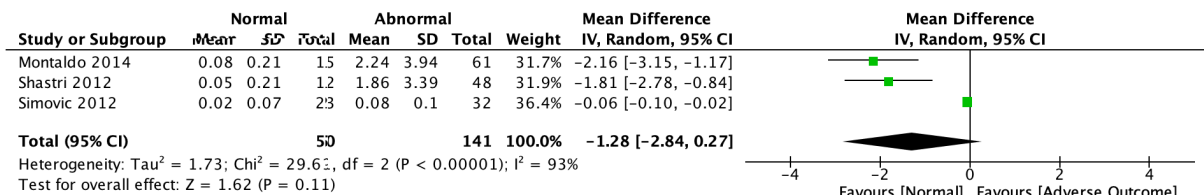
**Figure 60 Forest plot comparison of serum nucleated red blood cells between groups with normal and abnormal outcome in NE.** There was no significant difference in serum nucleated red blood cells between groups. The point estimate suggested that serum nRBC was 13.15 nRBC/mm<sup>3</sup> lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -9.56 nRBC/mm<sup>3</sup> to 35.86 nRBC/mm<sup>3</sup>.



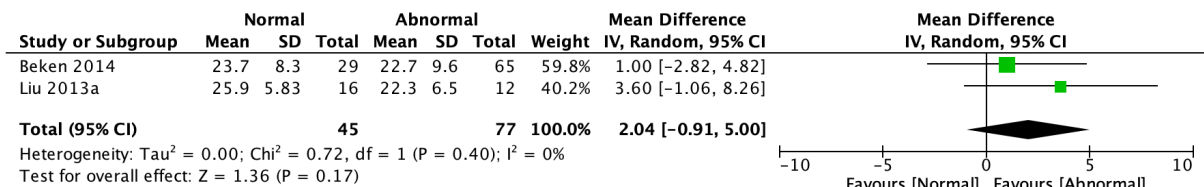
**Figure 61 Forest plot comparison of serum S100 between groups with normal and abnormal outcome in NE.** There was no significant difference in serum S100 between groups. The point estimate suggested that serum S100 was 10.65 ng/mL lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -1.48 ng/mL to 22.78 ng/mL.



**Figure 62 Forest plot comparison of serum TNF- $\alpha$  between groups with normal and abnormal outcome in NE.** There was no significant difference in serum TNF- $\alpha$  between groups. The point estimate suggested that serum TNF- $\alpha$  was 14.27 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -14.05 pg/ml to 42.6 pg/ml.



**Figure 63 Forest plot comparison of serum troponin between groups with normal and abnormal outcome in NE.** There was no significant difference in serum troponin between groups. The point estimate suggested that serum troponin was 1.28 pg/ml lower in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.27 pg/ml to 2.84 pg/ml.



**Figure 64 Forest plot comparison of serum white cell count between groups with normal and abnormal outcome in NE.** There was no significant difference in blood white cell count between groups. The point estimate suggested that serum WCC was 2.04 WCC/mm<sup>3</sup> higher in those with normal outcome compared to those with adverse outcome, however the 95% CI ranged from -0.91 WCC/mm<sup>3</sup> to 5.00 WCC/mm<sup>3</sup>.

## Discussion

We found that lower serum IL-6, IL-8, NSE, and TNF- $\alpha$  were associated with good long-term outcome in NE. However, the confidence intervals around the point estimate in the meta-analysis for all these biomarkers were very wide. Although the direction of the effect was consistent within each meta-analysis, confidence intervals of individual included studies were also very wide and crossed the line of no effect, and there was very limited overlap of confidence intervals between studies. Therefore, we are interpreting the association of higher in serum IL-6, IL-8, NSE, and TNF- $\alpha$  and an increased risk of abnormal outcome with extreme caution and providing very low-level evidence only. We suggest that the question requires further investigation. The inconsistency of results means it would not be appropriate to interpret these results as providing cut-off values for prognostic purposes for infants with NE.

We examined the association between cerebrospinal fluid (CSF) IL-1 $\beta$ , NSE, and TNF- $\alpha$  and outcome in infants with NE, however we did not find any significant associations. For CSF NSE 5 studies were included, 4 of which found lower NSE in infants with normal outcome however one outlier study found no significant difference between groups. This outlier was given significant weighting in the meta-analysis, and there was no methodological reason identified to exclude it from the meta-analysis. Therefore, we are interpreting the finding of no significant difference in CSF NSE between groups with extreme caution. It is possible given the consistency between studies that lower CSF NSE is associated with reduced risk of abnormal outcome, but we cannot make a definitive

conclusion from the current meta-analysis. We suggest that the question remains unanswered and requires further investigation.

We found an association between lower serum IL-6 and blood lactate levels was associated with good short-term outcome in infants with NE. The meta-analysis for IL-6 included 5 studies, of which 2 individual studies demonstrated a significant difference between groups. The confidence intervals for all other studies crossed the line of no effect and there was very limited overlap of confidence intervals. Therefore, we are interpreting the association of higher in serum IL-6 and an increased risk of abnormal short-term outcome with extreme caution and providing very low-level evidence only. We suggest that the question requires further investigation. There was excellent consistency of effect direction for blood lactate, with 3 of 4 included studies demonstrating a significant difference between group and a high degree of overlap between studies. However, the confidence interval around the point estimate for the meta-analysis was wide. Therefore, the inconsistency of results means it would not be appropriate to interpret these results as providing cut-off values for prognostic purposes for infants with NE.

This is the first systematic review to our knowledge to examine a wide range of blood, serum, and CSF biomarkers to predict outcome in NE in the era of therapeutic hypothermia. Ramaswamy et al. published a study in 2009 that demonstrated similar results to our study [106]. They found that serum IL-1 $\beta$ , serum IL-6, CSF IL-1 $\beta$ , and CSF NSE were putative predictors of long-term outcome in infants with NE. However, this was prior to the introduction of routine treatment with TH in high-resource settings.

Despite large volume of work and resources have been applied to the understanding of blood, serum, and CSF biomarkers in NE, the current evidence for any individual biomarker remains very limited. A very large number of studies could not be included in a meta-analysis despite providing useful prognostic information due to variations in reporting mechanisms and a standardised approach to measurement and reporting of biomarkers in NE would improve this issue. The EQUATOR Network provides a framework for reporting and following its recommendations would result in greater certainty of evidence from meta-analyses.

The prognostic information from blood, serum, and CSF biomarkers does not compare favourably to other prognostic markers such as MRI (Chapter 3). There is much greater consistency of prognostic information from MRI and MRS compared to any biomarker in this study. However, biomarkers provide additional information regarding the pathogenesis of the condition.

Biomarkers not only provide prognostic outcomes but can also provide improved understanding of the underlying pathophysiology of the condition, which remains incompletely understood. Much of our understanding of the pathophysiology of NE comes from pre-clinical models [420]. However, many pre-clinical models involve ligation of the carotid artery in rodents or post-natal systemic hypoxia insults in piglets. While these models have vastly improved our understanding of the pathophysiology of the condition,

they may not completely reflect the global hypoxic insult that human neonates with NE experience in the perinatal period. It would aid our understanding of the pathophysiology of NE if we could accurately correlate the mechanisms in pre-clinical models and human neonates diagnosed with NE and it could also lead to faster development of adjunctive therapies in NE. Measurement of inflammatory biomarkers in patients with NE will help with this to assess if the pre-clinical model accurately reflects the pathophysiology in NE but will likely require examination of samples from a very large cohort of patients. Despite the limitations of combining studies in a meta-analysis this may be the most pragmatic method to improve our understanding.

Unfortunately, the studies included in this systematic review are too small and there is too much variability between studies in timing of measurement and the characteristics of included patients to draw any significant conclusions. Another limitation of this systematic review is that it is difficult to capture the complexity of the different phases of injury in NE, as timing of blood sampling varied between each study. There were an insufficient number of included studies and insufficient consistency of sampling timing between studies to complete a subgroup analysis by timing. Biomarkers are also influenced by presence of infection, duration of hypoxia, gender, time of day when sampling completed, whether the infant is undergoing TH, other medications used in their treatment, and this is not represented in this systematic review.

## Conclusion

We found evidence of an association between lower IL-6, IL-8, NSE, and TNF- $\alpha$  and good long-term outcome, and between lower IL-6 and blood lactate and good short-term outcome in NE. However, the results from each meta-analysis only provided very-low certainty evidence. These biomarkers should be the focus of future studies of prognostic biomarkers in NE.



## Chapter 5: Cochrane review: melatonin treatment for newborns with neonatal encephalopathy

### Abstract

**Background:** Melatonin is a promising adjunct therapy to therapeutic hypothermia (TH) in neonatal encephalopathy (NE). It is a potent antioxidant, anti-inflammatory, and anti-apoptotic agent. It easily crosses the blood-brain barrier and has an excellent safety profile in neonates. Melatonin treatment as an adjunct to HT in animal models of NE have demonstrated reduced cell death and improved outcomes. We examined the current evidence for melatonin treatment in neonates with NE.

**Methods:** A comprehensive search was run on 21 October 2020 in the databases CENTRAL, MEDLINE, clinical trials databases, and the reference lists of retrieved articles for randomized controlled trials (RCTs) and quasi-RCTs. The review was limited to include only RCTs and quasi-RCTs that compared melatonin to standard treatment or placebo. The primary outcome was a reduction in death or adverse neurodevelopmental outcome at  $\geq 18$  months of age. Secondary outcomes included differences in mortality, neurodevelopmental disability (NDD) and abnormalities on MRI brain. 2 reviewers independently screened the literature search results, completed the data extraction and assessed the quality of included studies. Standard Cochrane methodologies were used throughout the review.

**Results:** The literature search identified 82 results of which 11 were suitable for full text screening. 4 RCTs of melatonin treatment in NE were identified. 2 compared melatonin as

an adjunct to HT and 2 compared melatonin monotherapy to placebo or standard treatment. All studies were judged to carry unclear risk of bias. All studies demonstrated improved outcomes with melatonin treatment, however there was significant variation in outcomes reported. Only 1 pilot study of 25 patients reported the primary outcome and did not detect any difference in death or NDD at 18 months, however it was not powered to detect a difference. The study reported improved cognitive outcomes for patients treated with melatonin compared to placebo. The review also identified 1 stage 3 RCT measuring the primary outcome, for which recruitment is ongoing.

**Discussion:** There is very low certainty evidence that melatonin may improve outcomes for patients with NE. Large stage 3 trials are urgently required as HT remains only partially effective in preventing adverse outcomes and further investigation of promising treatments should not be delayed.

## Introduction

### Description of the intervention

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring hormone which is primarily released by the pineal gland in the brain [78]. The release of melatonin is regulated by sensors in the eye which detect changes in light exposure. There is significant 24-hour variation in blood melatonin levels, with concentrations being lower in the day and higher at night. Melatonin is an important regulator of the circadian rhythm, but it also has anti-inflammatory [181], anti-oxidant [182], and anti-apoptotic [183] properties which make it a promising intervention for neuroprotection in NE.

Melatonin regulates the immune system in several ways. It acts as an anti-inflammatory molecule under conditions of exacerbated immune responses [421]. It exerts its influence on the innate immune system by regulating the lifespan of leukocytes by interfering with processes of cell death, regulating the release of pro-inflammatory cytokines and leukotrienes, and modulating the production of pro-inflammatory enzymes [186]. Melatonin is also a potent anti-oxidant. It indirectly stimulates the production of anti-oxidant enzymes including superoxide dismutase, glutathione peroxidase, and glutathione reductase, and inhibits the production of the pro-oxidant enzyme nitric oxide synthase [182]. Melatonin is a direct free radical scavenger, reducing the toxic effects of reactive oxygen species and leading to further reduction in oxidative stress [190]. Melatonin reacts with reactive oxygen species to form N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK). AFMK has potent anti-oxidant effects and scavenges radical oxygen species as the final

melatonin compound involved in the free radical scavenging cascade [422]. Melatonin exerts its effect via several different pathways. It readily crosses the blood-brain barrier and high levels accumulate in the central nervous system [184]. Because neurons have a high oxygen requirement, they are very vulnerable to oxidative stress. Some effects occur indirectly via two G protein-coupled receptors, M1 and M2, which are widely distributed throughout the body and the brain [423], but melatonin also works through its diverse anti-oxidative mechanisms which prevent free-radical-induced oxidative damage to the electron transport chain and mitochondrial DNA [182]. Melatonin is also believed to have a promising neuroprotective role through reduction of neuroinflammation [424]; and in models of endotoxaemia it has been found to regulate the pro- and anti-inflammatory cytokine network, modulate gene expression, and preserve mitochondrial integrity [425]. Several exogenous forms of ligands that attach to melatonin receptors are available and effective [426]. However, very high pharmacological doses of melatonin may be required to exert anti-oxidant activity. In the piglet model, the beneficial effects of melatonin have been found to be dose-dependent, with earlier achievement of therapeutic levels (15 mg/L to 30 mg/L) as important [427]. Melatonin can be administered by the enteral or intravenous route, although the recommended route of administration for acute neuroprotection in the newborn is intravenous as it results in greater bioavailability [428]. Supplementation with melatonin has demonstrated a rapid and sustained rise in plasma melatonin concentration in those undergoing TH [428]. Melatonin is primarily metabolised in the liver via cytochrome CYP1A2 enzyme; this enzyme is involved in the metabolism of a number of other commonly used medications such as paracetamol and caffeine, which may affect the bioavailability of

melatonin [429]. Melatonin has been studied in a number of conditions in the neonatal population and has an excellent safety profile [192].

#### How the intervention might work

Brain injury in NE results from the consequences of HIE, which include systemic inflammation, oxidative stress, and increased apoptotic activity [430] and ultimately result in cell death. Recent studies have shown that increased neutrophil activation [44] and elevated circulating pro-inflammatory cytokines [119] are associated with worse outcomes in NE. Clinical trials have demonstrated a reduction in circulating leukocytes and pro-inflammatory chemokines in patients treated with TH [107], suggesting that modulation of brain injury in NE occurs via immunosuppression. Further studies have demonstrated that administration of melatonin leads to a downregulation of neutrophil activity and pro-inflammatory cytokines in the neonatal population [187-189]. Newborns are more vulnerable to oxidative stress than older individuals [431], and oxidative stress is a critical contributor to brain injury in NE. Newborns with NE are unable to compensate for the excess production of reactive oxygen species following hypoxic or ischaemic insult. This in turn leads to an accumulation of toxins and further systemic inflammation which results in worsening brain injury. Studies of several conditions involving oxidative stress in newborns, including perinatal asphyxia, sepsis, and respiratory distress syndrome, have demonstrated a reduction in oxidative stress with melatonin supplementation [192]. In NE, oxidative stress combines with mitochondrial dysfunction to result in mitochondrial energy failure, a key regulator of apoptotic cell death [52]. Melatonin protects against neuron apoptosis via a

wide range of effects, including reduced release of cytochrome c, increased expression of anti-apoptotic proteins, and reduced expression of pro-apoptotic proteins. These actions help prevent excess mitochondrial permeability and stabilise the mitochondrial membrane potential [194], leading to reduced apoptotic cell death. Several animal models of NE have demonstrated improved outcomes with melatonin supplementation. Neonatal rats with HIE brain injuries were at lower risk of learning deficits and behavioural asymmetry when treated with melatonin [195]. Newborn mice with white matter brain lesions demonstrated improved secondary repair of these lesions when treated with melatonin [197]. A piglet model of HIE demonstrated improved neuroprotection as measured by magnetic resonance spectroscopy when melatonin treatment was combined with TH, compared to TH alone [196]. Lamb and rat models of NE have demonstrated that the neuroprotective effects of melatonin treatment are mediated through reduced pro-inflammatory markers, reduced apoptosis-mediated cell death and reduced cerebral oxidative stress [432, 433]. Melatonin has an excellent safety profile: it is well tolerated both in high doses by adults, with no adverse effects on sedation [434], and by children on long-term treatment, with no suppression of endogenous melatonin secretion [435]. It has been studied in many conditions in the neonatal population, including respiratory distress syndrome [188] and neonatal sepsis [436], with no adverse effects reported. The largest safety study of melatonin in the neonatal population, a retrospective study of 85 neonatal patients enrolled in multiple clinical trials of different conditions, demonstrated improved clinical outcomes and no adverse events [198]. Studies have not demonstrated any evidence of toxicity, even when administered at very high doses [198]. Enteral and intravenous administration of

melatonin are feasible and have demonstrated increased plasma concentration of melatonin. It has also been shown following melatonin administration, neonates have reduced clearance and prolonged half-life compared to adults. Hypothermia does not appear to cause any significant changes in pharmacokinetics in the neonatal population [428], therefore melatonin could make a good adjunct to TH.

### Aims

To assess the current evidence for benefits and harms of melatonin use in patients with neonatal encephalopathy.

### Hypothesis

We hypothesised that melatonin treatment would demonstrate improved outcomes in participants with neonatal encephalopathy.

## Methods

Criteria for considering studies for this review

### *Types of studies*

We included all randomised and quasi-randomised controlled trials comparing the use of melatonin with standard care for patients with neonatal encephalopathy.

### *Types of participants*

We included infants born at or beyond 35 weeks' gestation, with no major congenital malformations at birth and meeting any of the following criteria.

1. Evidence of perinatal asphyxia, with each enrolled infant satisfying at least one of the following criteria
  - a. Apgar score of five or less at 10 minutes
  - b. Mechanical ventilation or resuscitation at 10 minutes
  - c. Cord pH of less than 7.1, or an arterial pH of less than 7.1 or base deficit of 12 or more within 60 minutes of birth
2. Evidence of encephalopathy according to Sarnat staging [437]
  - a. Stage 1 (mild): hyperalertness, hyperreflexia, dilated pupils, tachycardia, absence of seizures
  - b. Stage 2 (moderate): lethargy, hyperreflexia, miosis, bradycardia, seizures, hypotonia with weak suck and Moro



c. Stage 3 (severe): stupor, flaccidity, small to mid-position pupils that react poorly to light, decreased stretch reflexes, hypothermia and absent Moro reflex

### 3. Severity of encephalopathy according to Thompson Score [289]

- a. Score 0 to 10 (mild)
- b. Score 11 to 14 (moderate)
- c. Score 15 or more (severe)

#### *Types of interventions*

The intervention is melatonin supplementation, by oral or intravenous administration, given to patients with NE within the first week of life. The comparison is with standard treatment for NE, including TH.

#### *Types of outcome measures*

##### Primary outcomes

Composite of death or long-term major neurodevelopmental disability, defined as a diagnosis of cerebral palsy (CP); Gross Motor Function Classification System (GMFCS) score of three or more; developmental delay, defined as a score on any standardised infant developmental assessment of more than two standard deviations below the mean; intellectual disability, defined as intelligence quotient (IQ) of more than two standard deviations below the mean; visual impairment, defined as visual acuity of less than 6/60 in both eyes; sensorineural hearing loss, defined as unable to hear sounds less than 40 decibels; or the need for more than one anticonvulsant medication for seizure control.

Long-term outcomes were reported for all studies that have evaluated children after 18 months of age.

## Secondary outcomes

### 1. Mortality

- a. Early (within the first week of life)
- b. Late (within the first year of life)
- c. At 18 months or final follow-up assessment

2. Each component of major neurodevelopmental disability assessed at more than 18 months of age

- a. Cerebral palsy [438]
- b. Gross Motor Function Classification System of three or more [439]
- c. Developmental delay [440]: defined as a score on any standardised infant developmental assessment of more than two standard deviations below the mean
- d. Intellectual disability [441]: defined as IQ of more than two standard deviations below the mean
- e. Visual impairment [442]: defined as visual acuity of less than 6/60 in both eyes
- f. Sensorineural hearing impairment [443]: defined as unable to hear sounds less than 40 decibels

3. Abnormal magnetic resonance imaging [309]

4. Multiorgan dysfunction [405]

5. Use of anti-convulsant medications

Search methods for identification of studies

### *Electronic searches*

We conducted a comprehensive search in October 2020 including: Cochrane Central Register of Controlled Trials (CENTRAL 2020, Issue 10) in the Cochrane Library and Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) (1946 to 21 October 2020). We have included the search strategies for each database in Table 17. We did not apply language restrictions. We searched clinical trial registries for ongoing or recently completed trials. We searched for trials from The World Health Organization's International Clinical Trials Registry Platform (ICTRP) ([www.who.int/ictrp/search/en/](http://www.who.int/ictrp/search/en/)) and the U.S. National Library of Medicine's ClinicalTrials.gov ([clinicaltrials.gov](http://clinicaltrials.gov)) via Cochrane CENTRAL. Additionally, we searched the ISRCTN Registry (<http://www.isrctn.com/>) for any unique trials not found through the Cochrane CENTRAL search.

#### Search methods

The RCT filters have been created using Cochrane's highly sensitive search strategies for identifying randomised trials [444] . The neonatal filters were created and tested by the Cochrane Neonatal Information Specialist. As of July 2019, Cochrane Neonatal no longer searches Embase for its reviews. RCTs and controlled clinical trials (CCTs) from Embase are added to the Cochrane Central Register of Controlled Trials (CENTRAL) via a robust process (see How CENTRAL is created). Cochrane Neonatal has validated their searches to ensure that relevant Embase records are found while searching CENTRAL (Ovelman

2020). Also starting in July 2019, Cochrane Neonatal no longer searches for RCTs and CCTs on the following platforms: ClinicalTrials.gov or from The World Health Organization's International Clinical Trials Registry Platform (ICTRP), as records from both platforms are added to CENTRAL on a monthly basis (see How CENTRAL is created). Comprehensive search strategies are executed in CENTRAL to retrieve relevant records. The ISRCTN Registry (at [www.isrctn.com/](http://www.isrctn.com/), formerly Controlled-trials.com), is searched separately.

CENTRAL via CRS Web:

Date searched: 21 October 2020

Terms:

1 MESH DESCRIPTOR Melatonin EXPLODE ALL AND CENTRAL:TARGET

2 melatonin AND CENTRAL:TARGET

3 N-acetyl-5-methoxytryptamine AND CENTRAL:TARGET

4 #1 OR #2 OR #3

5 MESH DESCRIPTOR Asphyxia EXPLODE ALL AND CENTRAL:TARGET

6 MESH DESCRIPTOR Asphyxia Neonatorum EXPLODE ALL AND CENTRAL:TARGET

7 MESH DESCRIPTOR Hypoxia-Ischemia, Brain EXPLODE ALL AND CENTRAL:TARGET

8 MESH DESCRIPTOR Brain Ischemia EXPLODE ALL AND CENTRAL:TARGET

9 MESH DESCRIPTOR Hypoxia EXPLODE ALL AND CENTRAL:TARGET

10 MESH DESCRIPTOR Hypoxia, Brain EXPLODE ALL AND CENTRAL:TARGET

11 MESH DESCRIPTOR Brain Injuries EXPLODE ALL AND CENTRAL:TARGET

12 brain injury or brain injuries AND CENTRAL:TARGET

13 neuroprotect\* or neuro-protect\* or neuro-restorative or neurorestorative AND CENTRAL:TARGET

14 HIE AND CENTRAL:TARGET

15 encephalopath\* AND CENTRAL:TARGET

16 hypoxi\* ADJ2 ischaemi\* AND CENTRAL:TARGET

17 hypoxi\* ADJ2 ischemi\* AND CENTRAL:TARGET

18 asphyxia\* AND CENTRAL:TARGET

19 anoxi\* ADJ2 ischemi\* AND CENTRAL:TARGET

20 anoxi\* ADJ2 ischaemi\* AND CENTRAL:TARGET

21 #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20

22 MESH DESCRIPTOR Infant, Newborn EXPLODE ALL AND CENTRAL:TARGET

23 infant or infants or infant's or "infant s" or infantile or infancy or newborn\* or "new born" or "new borns" or "newly born" or neonat\* or baby\* or babies or premature or prematures or prematurity or preterm or preterms or "pre term" or premies or "low birth weight" or "low birthweight" or VLBW or LBW or ELBW or NICU AND CENTRAL:TARGET

24 #23 OR #22 AND CENTRAL:TARGET

25 #24 AND #21 AND #4

MEDLINE via Ovid:

Date ranges: 1946 to 21 October 2020

Terms:

1. exp Melatonin/
2. melatonin.mp.
3. N-acetyl-5-methoxytryptamine.mp.
4. 1 or 2 or 3
5. exp Asphyxia/
6. exp Asphyxia Neonatorum/
7. exp Hypoxia-Ischemia, Brain/
8. exp Brain Ischemia/
9. exp Hypoxia/
10. exp Hypoxia, Brain/
11. exp Brain Injuries/
12. (brain injury or brain injuries).mp.
13. (neuroprotect\* or neuro-protect\* or neuro-restorative or neurorestorative).mp.
14. HIE.tw,kf.
15. encephalopath\*.mp.
16. (hypoxi\* adj2 ischaemi\*).mp.
17. (hypoxi\* adj2 ischemi\*).mp.
18. asphyxia\*.mp.
19. (anoxi\* adj2 ischemi\*).mp.

20. (anoxi\* adj2 ischaemi\*).mp.
21. or/5-20
22. exp infant, newborn/
23. (newborn\* or new born or new borns or newly born or baby\* or babies or premature or prematurity or preterm or pre term or low birth weight or low birthweight or VLBW or LBW or infant or infants or 'infant s' or infant's or infantile or infancy or neonat\*).ti,ab.
24. 22 or 23
25. randomized controlled trial.pt.
26. controlled clinical trial.pt.
27. randomized.ab.
28. placebo.ab.
29. drug therapy.fs.
30. randomly.ab.
31. trial.ab.
32. groups.ab.
33. or/25-32
34. exp animals/ not humans.sh.
35. 33 not 34
36. 24 and 35
37. randomi?ed.ti,ab.
38. randomly.ti,ab.

39. trial.ti,ab.

40. groups.ti,ab.

41. ((single or doubl\* or tripl\* or treb\*) and (blind\* or mask\*)).ti,ab.

42. placebo\*.ti,ab.

43. 37 or 38 or 39 or 40 or 41 or 42

44. 23 and 43

45. limit 44 to yr="2019 -Current"

46. 36 or 45

47. 4 and 21 and 46

ISRCTN:

Date searched: 21 October 2020

Terms:

Interventions: Melatonin AND Participant age range: Neonate

Melatonin within Participant age range: Neonate

Interventions: N-acetyl-5-methoxytryptamine AND Participant age range: Neonate

*Table 17 Search terms for used for this Cochrane review.*

### *Searching other resources*

Review authors cross-referenced relevant literature including identified trials and existing review articles. We searched the reference lists of previous reviews, and trials



included in the current review, for citations and cross-references. We searched abstracts from conference proceedings, including those of the joint European Neonatal Societies/European Society for Paediatric Research (jENS/ESPR) (1960 to present), the Pediatric Academic Societies (PAS) (1998 to present) and the Perinatal Society of Australia and New Zealand (PSANZ) (1998 to present). We also consulted experts in the subject area.

#### Data collection and analysis

We collected and analysed data in accordance with the standard methods of Cochrane Neonatal. In the following section, we report only the methods used in the review. Planned and unused methods can be found in the review Protocol [445].

#### *Selection of studies*

Two review authors (TH and SA) independently assessed the eligibility of all retrieved studies for inclusion in the review. Disagreements were resolved by discussion between the two review authors. We assessed studies in the usual consecutive format, starting with title and abstract screening and subsequently full-text screening. We used Covidence software to facilitate this process [446]. We documented the study selection process in a PRISMA flow diagram.

### *Data extraction and management*

Two review authors independently performed data extraction using a structured form. Differences were resolved by discussion between the two review authors. We contacted study authors to request additional data from unpublished studies in abstract form. All analyses were performed using Review Manager 5 software [447].

### *Assessment of risk of bias in included studies*

Two review authors (TH and SA) independently assessed all included trials using the Cochrane 'Risk of bias' tool. Each study was assigned a judgement of low, high, or unclear risk of bias for the following domains [444].

1. Sequence generation (selection bias)
2. Allocation concealment (selection bias)
3. Blinding of participants and personnel (performance bias)
4. Blinding of outcome assessment (detection bias)
5. Incomplete outcome data (attrition bias)
6. Selective reporting (reporting bias)
7. Any other bias

Disagreements were resolved by discussion or by consulting a third review author (EM). See Appendix for a more detailed description of risk of bias for each domain.

### *Measures of treatment effect*

We expressed treatment effects for dichotomous outcomes using summary odds ratio (OR) with 95% confidence intervals (CIs).

### *Unit of analysis issues*

Our primary outcome is a composite of dichotomous outcomes. Each constituent of the primary outcome is clearly defined by standardised criteria and expressed in standardised unit measurement. Where outcomes are expressed as continuous data, we used internationally accepted cut-off values to categorise participants, as outlined above.

### *Dealing with missing data*

Our strategy to deal with missing data followed Cochrane Handbook guidance [444], as follows.

1. Where possible, we will contact original investigators for missing data.
2. Where possible, missing standard deviations will be imputed using the coefficient of variation (CV), or calculated from other available statistics including standard errors, CIs, t values, and P values.
3. If the data are assumed to be missing at random, they will be analysed without imputing any missing values.

4. If data cannot be assumed to be missing at random then the missing outcomes will be imputed with replacement values, assuming all to have a poor outcome.
5. If we are required to impute data, we will make explicit the assumptions of any methods used.
6. We will perform sensitivity analyses to assess how sensitive results are to reasonable changes in the assumptions that are made.
7. We will address the potential impact of missing data on the findings of the review in the 'Discussion' section.

#### *Assessment of heterogeneity*

We estimated the treatment effects of individual trials and examine heterogeneity among trials by inspecting the forest plots and quantifying the impact of heterogeneity using the  $I^2$  statistic. We graded the degree of heterogeneity as: less than 25% no heterogeneity; 25% to 49% low heterogeneity; 50% to 75% moderate heterogeneity; more than 75% substantial heterogeneity. If we note statistical heterogeneity ( $I^2 > 50\%$ ), we will explore the possible causes (e.g. differences in study quality, participants, intervention regimens, or outcome assessments).

#### *Assessment of reporting biases*

We compared the reported measured outcomes to trial protocols where available, and the methods section of each trial with the results reported. We were unable to draw

funnel plots and test for funnel plot asymmetry, because there were too few included studies.

### *Data synthesis*

We performed a meta-analysis of some secondary outcomes. We used standardised methodologies as described in the Cochrane Handbook [444]. Meta-analysis will be conducted using the inverse variance method for continuous outcomes, and the Mantel-Haenzel method for dichotomous outcomes. We used the random-effects model and present all our results with 95% CIs. We calculated the RR, RD, and NNTB or NNTH if the RD is significant — each with 95% CIs — for categorical outcomes. We calculated the MD with 95% CIs for continuous outcomes. Where continuous outcomes are measured using different scales, we planned to express the treatment effect as SMD with 95% CIs. For any outcomes where the included studies are not sufficiently homogeneous, or where insufficient data are available for meta-analysis, we will present a narrative synthesis.

### *Certainty of evidence*

We will use the GRADE approach, as outlined in the GRADE Handbook [448], to assess the certainty of evidence for the following (clinically relevant) outcomes: mortality, presence of cerebral palsy, developmental delay or intellectual disability, visual impairment or sensorineural hearing loss, abnormal magnetic resonance imaging, multiorgan dysfunction, and use of anti-convulsant medications. Two review authors will independently assess the certainty of the evidence for each of the outcomes above. We will consider evidence from randomised controlled trials as high certainty, but will downgrade the

evidence by one level for serious (or two levels for very serious) limitations based upon the following: design (risk of bias), consistency across studies, directness of the evidence, precision of estimates, and presence of publication bias. We will use GRADEpro GDT (GRADEpro GDT) to create a ‘Summary of findings’ table to report the certainty of the evidence. The GRADE approach results in an assessment of the certainty of a body of evidence, of one of the following four grades.

1. High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
2. Moderate certainty: we are moderately confident in the effect estimate; the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
3. Low certainty: our confidence in the effect estimate is limited; the true effect may be substantially different from the estimate of the effect.
4. Very low certainty: we have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of effect.

#### *Subgroup analysis and investigation of heterogeneity*

We conducted a subgroup analysis based on the adjunct use of TH.

#### *Sensitivity analysis*

We did not perform a sensitivity analysis due to insufficient included studies.

## Results

### Description of studies

See Characteristics of included studies and Characteristics of excluded studies tables.

### Results of the search

Our literature search identified 80 records. A further single record was identified through a search of the grey literature, and we identified a single ongoing study from a search of clinical trial registers. 73 records remained after exclusion of duplicates. Two review authors (TH and SA) screened the remaining records for inclusion using Covidence software. We excluded 62 records during title and abstract screening leaving 11 records for which we retrieved the full texts for further examination. During the full text screening we excluded five records due to ineligible study designs, one record due to ineligible comparator intervention, and one record as it is a duplicate of an included study (Criteria for considering studies for this review); see Characteristics of excluded studies tables. Of the four included records, only one study reported the primary outcome for this review, see Figure 1.

### Included studies

We included four studies (155 participants) in this review (Ahmad; Aly; Fulia; Jerez-Calero). See Characteristics of included studies for full details.

### *Study design*

All four included studies were randomised controlled trials (RCT) designed to assess the efficacy and safety of melatonin use in children with neonatal encephalopathy. Two studies were placebo-controlled (Fulia; Jerez-Calero), and one study was double-blinded (Jerez-Calero). Two studies compared melatonin as an adjunct therapy to TH (Aly; Jerez-Calero) and two studies compared melatonin to standard therapy alone (Ahmad; Fulia).

### *Settings*

All four studies were conducted in hospital environments, with one study completed in each of Pakistan, Egypt, Italy and Spain.

### *Participants*

All four studies recruited late-preterm or term newborns diagnosed with NE or HIE diagnosed within the first 12 hours of life.

Ahmad recruited participants with gestational age of at least 34 weeks and diagnosed with HIE within the first 12 hours of life. The diagnosis of HIE was based on a case definition of HIE which included clinical criteria only, and which is not referenced in the study. The severity of HIE was assessed using the Thompson score. Patients with features other than HIE at admission were excluded, although those features are not specified in the publication. 80 participants were included in the study, with 40 included in both the melatonin treatment and placebo or standard treatment groups. There was no significant



difference in gestational age or severity of HIE between participants in the melatonin treatment and placebo or standard treatment groups. Participants with mild NE were included in the study and accounted for 10% of the standard treatment group, and 15% of the melatonin treatment group.

Aly recruited 30 inborn patients with gestational age of at least 38 weeks and diagnosed with HIE within the first 6 hours of life. Inclusion criteria included Apgar score, evidence of metabolic or mixed acidosis on initial analysis, and evidence of moderate or moderately severe encephalopathy. Participants were excluded for a variety of reasons including evidence of infection, low birthweight, or if there was evidence of congenital or chromosomal abnormality. Patients presenting in extremis were also excluded from participation in this study. There was a higher proportion of patients with severe encephalopathy in the standard treatment group (27%) compared to the melatonin treatment group (13%), however this difference was not statistically significant ( $p = 0.65$ ), and a higher proportion of patients in the standard treatment group that required inotropic support (27%) compared to the melatonin treatment group (7%), but again this difference was not statistically significant ( $p = 0.13$ ).

Fulia recruited 20 participants with perinatal asphyxia diagnosed within the first 6 hours of life. The diagnosis of perinatal asphyxia was based on the five-minute Apgar score and evidence of systemic acidosis at birth. No neurological criteria or examination was required for the diagnosis. No threshold minimum gestational age at birth is specified in the

study, however all participants were at least 38 weeks gestation at birth. Participants were excluded from the study if they had evidence of an infectious disease, a congenital malformation of the brain, or an inborn error of metabolism. 16 participants were diagnosed with severe asphyxia, and the identified causes in all cases were a tight nuchal cord or placental abruption. In the other 4 cases mild asphyxia was diagnosed and no cause was identified. There is no further information provided regarding the proportion of mild or severely asphyxiated participants in either treatment group for this study. The study provides a comparison of clinical data for the total group of asphyxiated participants with a group of healthy control participants, but no comparison between the group of asphyxiated participants treated with melatonin and the group treated with placebo.

Jerez-Calero recruited 25 participants, 12 in the hypothermia plus melatonin (HM) group and 13 in the hypothermia plus placebo (HP) group. Newborns were considered for recruited to the RCT if they met international criteria for therapeutic hypothermia treatment. Inclusion criteria for the study were gestational age of at least 36 weeks at birth; severe perinatal asphyxia based on Apgar score, the need for resuscitation at birth, and evidence of metabolic acidosis; and a diagnosis of moderate or severe encephalopathy based on the modified Sarnat and Sarnat score. Participants were excluded if they had birthweight less 1800gms, surgical pathology within the first three days of life, a serious congenital malformation within the CNS, hypoxemia unresponsive to mechanical ventilation, severe coagulopathy, chromosomal abnormality, or if they could not be recruited within 6 hours of postnatal life. Participants in the HP group had more severe

encephalopathy measured by higher mean Sarnat score ( $20.69 \pm 10.67$ ) compared to the HM group ( $12.72 \pm 7.57$ ).

### *Interventions*

All included studies provided different doses of melatonin supplementation. Jerez-Calero is the only trial to provide melatonin via the intravenous (IV) route. Participants in the melatonin treatment group received 5mg per kilogram of melatonin daily for first three days of life. Melatonin at a concentration of 6.5mg/mL was dissolved in propylene glycol and macrogol and administered by infusion over two hours. Two studies provided melatonin via the enteral route. In the Aly trial, participants in the melatonin treatment group were administered 10mg per kg daily for five days. Melatonin tablets were crushed and dissolved in distilled water prior to administration via orogastric tubes. Ahmad administered a single 10mg dose of melatonin via a nasogastric tube on admission. No further details of the intervention are provided. In the Fulia trial, participants in the intervention group received a total of 80mg of melatonin, administered in 8 doses of 10mg every 2 hours. Melatonin was dissolved in 1:90 ratio of ethanol:physiological saline, however the route of administration is not specified.

Two trials (Aly; Jerez-Calero) provided melatonin as an adjunctive therapy to therapeutic hypothermia, with participants in both the melatonin treatment and placebo or standard treatment groups receiving TH in both trials. Aly trial provided TH by removing the heat source, exposure to ambient temperature and application of ice packs to the

participants chest, head, and shoulders while monitoring deep rectal temperature every 30 minutes to ensure the participants core temperature remained between 33-34°C. TH was continued up to 72 hours, and participants were then rewarmed by 0.5°C every hour until core temperature reached 36.5°C. Jerez-Calero also provided whole-body hypothermia, however an automated cooling device was specified in the study. The target temperature of 33-34°C was maintained for 72 hours using Tecotherm Neo automatic device. Rectal temperature was monitored every two seconds using Servo-controlled technology to ensure temperature remained stable. Participants were rewarmed by 0.5°C per hour until 36.5°C was reached.

The other two included studies specified that both groups received standard care. In the trial by Ahmad, this included treatment for participants in both groups of oxygen therapy, IV fluids, invasive monitoring, broad spectrum antibiotics and control of fits if required, although the medications provided are not specified. Participants in the Fulia trial received care in the NICU if required, with monitoring, assisted ventilation and other intensive care procedures were available. They also received their mother's milk or human milk from a milk bank without iron supplementation.

### *Controls/Comparators*

Two included studies compared melatonin treatment to placebo (Fulia, Jerez-Calero). The Fulia trial provided an equal volume of the diluent (1:90 ethanol:physiological saline) to participants in the placebo group. In the trial by Jerez-Calero, participants in the

placebo group received the same volume of infusion over 2 hours, however 0.9% normal saline was provided instead of melatonin suspension.

The other two studies compared melatonin treatment to standard care (Ahmad, Aly). Aside from the difference in the intervention, there were no differences in treatment between melatonin treatment and placebo or standard treatment groups in any of the four included studies.

### *Outcomes*

#### *Primary outcome*

One of the studies measured and reported the primary outcome of mortality and long-term major neurodevelopmental neurodisability (NDD) (Jerez-Calero). The study reported mortality up to 18 months of age and measured NDD by Gross Motor Function Classification System (GMFCS), Tardieu scale, and Bayley Scales of Infant and Toddler development (Bayley-III) at 6 and 18 months of age. They compared outcomes between groups treated with therapeutic hypothermia plus melatonin (HM) and therapeutic hypothermia plus placebo (HP). Between-group differences in the incidence of mortality, high-grade GMFCS classification, and high-grade Tardieu scale were reported by significance testing only. Differences in Bayley-III were reported as group means and standard deviations, and by significance testing.

## Secondary outcomes

### Mortality

All studies reported mortality outcomes for participants recruited, however the duration of follow up varied between each study. Fulia reported the shortest follow up duration of 3 days, Ahmad reported follow up to 28 days, Aly reported follow up to 6 months, and Jerez-Calero reported follow up to 18 months.

### Long-term neurodevelopmental disability

One study measured NDD at 6 and 18 months of age, and this is considered above as a component of the primary outcome (Jerez-Calero). One other study also reported mortality and neurodevelopmental outcomes at six months of age (Aly). Participants were evaluated by neurological examination and by the Denver Developmental screening test (DDST-II) which examined development in four major categories: gross motor, language, fine motor adaptive, and personal-social. Participants were scored in each category as advanced, normal, caution or delayed and an adverse outcome was considered to be  $\geq 2$  delays, questionable if there were one delay and/or  $\geq 2$  cautions and normal if there were no delays and a maximum of one caution.

### Abnormal MRI brain

Participants in the studies by Aly and Jerez-Calero had neuroimaging outcomes measured by magnetic resonance imaging (MRI) brain. After 2 weeks and once clinically stable, participants in the Aly study had T1 and T2 weighted images obtained on a 1.5T MRI

machine. The posterior limb of the internal capsule (PLIC) was assessed and graded as normal, abnormal, or equivocal. The basal ganglia and thalami (BG/T) were assessed and graded as normal or abnormal with minimal, moderate, or severe abnormalities according to Barkovich scoring system. White matter (WM) changes were assessed, and their presence was documented for the lobe involved and for the presence of haemorrhagic changes. Haemorrhagic WM changes were graded as moderate or severe. All MRI images were reviewed by a radiologist blinded to treatment groups. Participants in the Jerez-Calero study had MRI brain at 1 week of age on a 1.5T MRI machine. The PLIC and BG/T were evaluated for the presence of abnormalities. If identified, abnormalities were graded as mild or moderate-severe. WM changes were also evaluated and graded as normal/minimal or moderate-severe. MRI images were assessed by a paediatric radiologist blinded to treatment groups.

#### Multi-organ dysfunction

No included study measured or reported this outcome.

#### Use of anti-convulsant medications

No included study measured or reported this outcome.

#### Other outcomes reported in at least one study

Two studies measured and reported the effects of melatonin treatment on electroencephalographic changes (Aly, Jerez-Calero). Unfortunately, the methodology varied significantly between the two studies and the outcomes could not be compared. Aly

measured 16-channel electroencephalogram (EEG) changes at enrolment and repeated the exam at two weeks of age. EEG tracings were categorised as continuous or discontinuous, with discontinuous tracings graded as moderate, severe or extreme. The presence of electrographic seizures on follow-up EEG are also reported. EEG tracings were reviewed by a neurologist that was blinded to treatment group. Jerez-Calero evaluated electroencephalographic features using continuous amplitude-integrated electroencephalography (aEEG) from admission to completion of the rewarming phase of therapeutic hypothermia. aEEG tracings were categories into five background patterns based on continuity and baseline voltages. The aEEG tracings were assessed by a NICU neonatologist blinded to treatment group.

#### Funding sources

The study by Jerez-Calero was supported by an award for independent clinical research from the Spanish ministry of health (EC11-222. EudraCT: 2012-000184-24). No sources of funding were reported for the other three included studies (Ahmad, Aly, Fulia).

#### Declarations of interest

Three studies stated that the authors had no conflicts of interest to declare (Ahmad, Aly, Jerez-Calero) and one study did not report whether the authors had any conflicts of interest (Fulia).



## Excluded studies

We excluded seven studies (seven full-text reports); for details, see the Characteristics of excluded studies tables. We excluded five studies due to ineligible study designs for this review, one study due to ineligible comparator intervention, and one study as it was a duplicate report of an included study.

## Risk of bias in included studies

We assessed the risk of bias in all four studies using the Cochrane RoB tool (Higgins 2011a; Higgins 2011b). We judged all four studies to have an overall unclear risk of bias as all studies included at least one domain that we judged as unclear risk of bias and no study included a domain that we judged as high risk of bias. We summarise our judgements below, and graphically in Figure 2 and Figure 3. A full account of our assessment can be found in the risk of bias tables, included with the Characteristics of included studies tables.

## *Allocation*

### Random sequence generation

One study reported using a robust randomisation process by using computer-generated numbers (Ahmad). There was insufficient detail regarding the randomisation process to allow a judgement of low or high risk of bias in the other included studies and all were judged as unclear risk of bias (Aly, Fulia, Jerez-Calero).

## Allocation concealment

We judged one study to be at low risk of bias in allocation concealment as a pharmacy-controlled randomisation process was employed for the study (Jerez-Calero). There is insufficient detail provided in the publications of the other studies to make an assessment of the methodology employed for allocation concealment and all were judged as unclear risk of bias (Ahmad, Aly, Fulia).

## Blinding

### Blinding of participants and personnel

One study employed robust methodology to ensure blinding of participants and investigators and we judged it to be at low risk of bias (Jerez-Calero). Two further studies did not blind or did not describe blinding participants or personnel, however we judged them to be at low risk of bias as the outcome was unlikely to be influenced by the lack of blinding (Ahmad, Fulia). We judged one study to be at unclear risk of bias as insufficient detail of the methodology employed in the blinding process was reported and some outcomes were at risk of being influenced by the lack of blinding (Aly).

### Blinding of outcome assessment

All studies were judged to be at low risk of bias. We judged two studies to be at low risk as all outcome assessors were masked to the treatment group during assessment (Aly, Jerez-Calero) and two studies did not blind or did not describe blinding the outcome

assessors, however the outcome was unlikely to be influenced by the lack of blinding (Ahmad, Fulia).

#### *Incomplete outcome data*

We judged one study to be at unclear risk of bias as participants who left against medical advice or did not complete the study period were excluded and there are no further details provided nor any discussion regarding the statistical methods employed for outcome analysis (Ahmad). The three remaining included studies reported all outcomes in full and there were very low rates of loss to follow up in each, so we judged them to be at low risk of bias.

#### *Selective reporting*

All studies were judged to be at low risk of bias. Two studies published protocols prior to commencement of the trial and reported all outcomes in the publication (Aly, Jerez-Calero). Two studies did not publish trial protocols, however it was clear from comparison with the published methods that the publication contains all measured outcomes and they are reported in full (Ahmad, Fulia).

#### *Other potential sources of bias*

We did not identify any further sources of bias in any of the included studies, so we judged all of them to be at low risk of bias.

Effects of the interventions

See: Summary of findings 1 Melatonin treatment compared to placebo or standard treatment for children with neonatal encephalopathy.

Summary of findings:

**Melatonin compared to standard therapy for newborns with neonatal encephalopathy**

**Patient or population:** newborns with neonatal encephalopathy  
**Setting:** hospital  
**Intervention:** melatonin (PO or IV)  
**Comparison:** standard therapy

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	N <sup>o</sup> of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with standard therapy	Risk with melatonin				
Death or major disability in survivors	Jerez-Calero is the only study to report the effects of the intervention on the primary outcome of death or long-term major NDD. The 2 outcomes are presented separately. There was no difference in mortality nor in major long-term NDD between groups. This is a pilot study and the sample size was insufficient to measure the effect of the intervention on the primary outcome.			25 (1 RCT)	⊕○○○ VERY LOW <sup>a,b</sup>	
Mortality follow up: 1 month	282 per 1,000	<b>96 per 1,000</b> (41 to 211)	<b>OR 0.27</b> (0.11 to 0.68)	155 (4 RCTs)	⊕○○○ VERY LOW <sup>b,c,d</sup>	
Neurodevelopmental disability follow up: 18 months	Jerez-Calero is the only study to report major NDD follow up to 18 months. There was no significant difference between groups in GMFCS classification or Tardieu scale. Only results of significance tests are presented, with no further information supplied. However, participants in the intervention group had significantly higher cognitive function (101.25 ± 21.51) at 18 months of age on BSID-III compared to the control group (85.56 ± 17.40). Participants in the intervention group also had higher language function (95.38 ± 24.47) compared to controls (83.22 ± 19.23) although the difference was not statistically significant (p = 0.06). Participants in the intervention groups also had higher motor function (96.13 ± 22.08) compared to controls (89.33 ± 26.12), although again the difference was not statistically significant (p = 0.35). This is a pilot study and the sample size was insufficient to measure the effect of the intervention on the incidence of major long-term NDD.			25 (1 RCT)	⊕○○○ VERY LOW <sup>a,b</sup>	
MRI abnormalities in PLIC or basal ganglia and thalamus	458 per 1,000	<b>477 per 1,000</b> (228 to 741)	<b>OR 1.08</b> (0.35 to 3.39)	50 (2 RCTs)	⊕○○○ VERY LOW <sup>b,e</sup>	
MRI abnormalities in the white matter	583 per 1,000	<b>322 per 1,000</b> (101 to 652)	<b>OR 0.34</b> (0.08 to 1.34)	50 (2 RCTs)	⊕○○○ VERY LOW <sup>b,e</sup>	
Multiorgan dysfunction				(0 studies)	-	Not measured in any trial.
Use of anticonvulsant medication				(0 studies)	-	Not measured in any trial.

\*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: Confidence interval; OR: Odds ratio; MD: Mean difference; PO: Per os (oral administration); IV: Intravenous; NDD: Neurodevelopmental disability; GMFCS: Gross motor function classification system

**GRADE Working Group grades of evidence**

**High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect

**Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different

**Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect

**Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

**Explanations**

- a. Downgraded two levels due to very serious imprecision (very small sample size and only the results of significance tests are presented)
- b. Downgraded one level due to study limitations (risk of bias)
- c. Downgraded one level due to imprecision (there are very few events in 3 of the 4 included studies)
- d. Downgraded one level due to inconsistency (subgroup analysis indicated no treatment effect for studies that treated participants with therapeutic hypothermia and the CI includes appreciable benefit and harm)
- e. Downgraded two levels for very serious imprecision (there are few events from two small sample RCTs, and the CI includes appreciable benefit and harm)

Unfortunately, as only one study reported the primary outcome for this systematic review, we were unable to combine results in a meta-analysis; therefore, we can only report the individual study outcomes.

### *Primary outcome*

#### *Mortality or long-term major neurodevelopmental disability*

Adverse outcome for this Cochrane review is defined as death or long-term abnormal neurodevelopmental outcome. This outcome was measured and reported in Jerez-Calero which measured neurodevelopmental outcome at 18 months of age by Bayley Scales of Infant and Toddler Development (Bayley-III), Gross Motor Function Classification System (GMFCS) classification, or Tardieu scale. No other included study measured long-term neurodevelopmental outcome.

Jerez-Calero recruited 25 participants who were randomised to the hypothermia plus melatonin (HM) group (N = 12) or the hypothermia plus placebo (HP) group (N = 13). The HP group had greater severity of NE, measured by mean Sarnat score, compared to the HM group, although the difference was not significant ( $p = 0.09$ ). No between-group differences in mortality or long-term neurodevelopmental disability were reported in the study, however this was a pilot study, and the sample size of the study was not powered to detect a difference. There was no between-group difference in mortality, one participant in both groups died. There was no difference in the incidence of higher GMFCS classification between groups ( $p = 1.00$ ), nor in higher grade of Tardieu scale ( $p = 0.58$ ). The results of

Bayley-III are reported as means and standard deviation rather than the incidence of abnormal results, often considered greater than one or two standard deviations below the mean. Between group differences were compared using Mann–Whitney tests. Participants in the HM group had better cognitive ability at 18 months of age compared to HP group ( $p = 0.05$ ). There were no differences in language ( $p = 0.06$ ), nor motor abilities ( $p = 0.35$ ).

### *Secondary outcomes*

#### *Mortality (analysis 1)*

All four included studies measured differences in mortality between melatonin treatment and placebo or standard treatment groups, however there is significant variation in the duration of follow-up as noted above. Meta-analysis of the reported data for the four trials (155 participants) showed that melatonin supplementation may decrease mortality (OR 0.27 95% CI 0.11 to 0.68). Heterogeneity was low ( $I^2 = 0\%$ ). Using the GRADE method, we assessed the certainty of evidence as very low. Certainty of evidence was downgraded by one level for each of study limitations (risk of bias), imprecision, and inconsistency. Subgroup analysis indicated that there was no evidence of an effect of melatonin on mortality in studies that provided melatonin as an adjunctive therapy to TH (OR 0.39, 95% CI 0.06 to 2.37) and the confidence interval included appreciable benefits and harm. There was insufficient consistency across studies to conduct further subgroup analyses.

#### *Long-term major neurodevelopmental disability*

As discussed as part of the primary outcome above, Jerez-Calero evaluated NDD at 6 and 18 months of age. The study by Aly also assessed NDD at 6 months of age, although a

different measurement scale was employed. Due to differences in assessment methodology, the two studies could not be combined in a meta-analysis. From a developmental assessment at 6 months follow-up, Jerez-Calero reported no difference in cognitive, language, or motor cognitive abilities on Bayley-III between participants in the HM group and those in the HP group. However, at six-month follow-up Aly reported higher incidence of normal neurological exam and developmental assessment in surviving patients treated with melatonin (71%) compared to those treated with standard care (27%). Overall, Aly reported increased survival without neurodevelopmental abnormalities at six months in the melatonin treated group compared to the standard treatment group ( $p = <0.001$ ).

#### Abnormal MRI brain (analysis 2 & 3)

We combined results of MRI brain evaluation into meta-analyses of the BG/T and WM separately. Both studies that reported outcomes of MRI brain evaluation divided results into these categories. There was no evidence of a difference in the incidence of BG/T abnormalities on MRI brain associated with melatonin treatment compared to standard treatment (OR 1.08, 95% CI 0.35 to 3.39, analysis 2). Nor was there evidence of a difference in the incidence of WM abnormalities on MRI brain associated with melatonin treatment compared to standard treatment (OR 0.3, 95% CI 0.02 to 4.43, analysis 3). We assessed the certainty of this evidence for both outcomes as very low, as certainty was downgraded in both by one level due to study limitations (risk of bias) and two levels due to very serious imprecision.



## Discussion

### Summary of main results

We evaluated the evidence for the use of melatonin treatment in patients with neonatal encephalopathy (NE). We included four studies (N= 155) that examined the effect of melatonin treatment compared to placebo or standard treatment to reduce mortality and long-term neurodevelopmental disability (NDD) in participants with NE. All four included studies demonstrated improved outcomes in at least one of the two components of the primary outcome with melatonin treatment compared to placebo or standard treatment, however the outcomes measured were different in each study.

We remain uncertain whether melatonin reduces the incidence of the combined outcome of mortality and long-term NDD. Only one study reported this primary outcome, so we could not complete a meta-analysis and a narrative summary was provided (Jerez-Calero). There was very low certainty evidence that compared to placebo, melatonin treatment did not reduce mortality or long-term NDD. This evidence however comes from a single pilot study with a very small incidence of adverse outcome, and it was not powered to detect a difference between groups.

Compared to placebo or standard treatment melatonin treatment may reduce mortality in patients with NE. A reduction in mortality is demonstrated only in studies that compared melatonin treatment to standard treatment without TH. No effect was

demonstrated in studies that compared melatonin treatment as an adjunctive therapy to TH compared to TH alone. We have very low certainty evidence, however, in reaching these conclusions.

We remain uncertain whether melatonin treatment improves neurodevelopmental outcomes. One small study reported neurodevelopmental outcomes up to 18 months of age in participants treated with melatonin as an adjunct to TH compared to placebo, and they demonstrated no reduction in long-term neurodevelopmental disability as measured by the standardised Gross Motor Functional Classification System and Tardieu scale, but they demonstrated improved cognitive outcomes on Bayley-III developmental assessment (Jerez-Calero). One further study measured neurodevelopmental outcomes to 6 months of age and demonstrated reduced abnormalities on neurodevelopmental screening measured by Denver Developmental Screening Test in patients treated with melatonin as an adjunct to the TH compared to TH alone (Aly).

There was very low certainty evidence that compared to placebo or standard treatment, melatonin treatment resulted in no differences in MRI brain outcome. There was no difference in the incidence of abnormalities in the thalamus, basal ganglia, or white matter on MRI brain (Aly; Jerez-Calero).

None of the included studies reported the effect of melatonin treatment compared to placebo or standard treatment on the incidence of multi-organ dysfunction or the use of anticonvulsant medications.

#### Overall completeness and applicability of evidence

There is currently insufficient evidence to prove or disprove the efficacy of melatonin treatment for patients with NE to improve outcome. Despite this lack of certainty melatonin appears to be a promising treatment as all studies demonstrated better outcomes in melatonin treatment groups compared to placebo or standard therapy in important outcomes including reduced mortality and better cognitive abilities.

The evidence is reassuring as no study reported adverse effects of melatonin treatment. Encouragingly, two of the included studies were completed in the past five years and both examined melatonin as an adjunctive therapy to TH and reported neurodevelopmental outcomes. We also identified one ongoing phase III study of melatonin as a adjunctive therapy to TH which will report long term ND outcomes up to 18 months of age (MELPRO), and one further phase I study which will evaluate ND outcomes at 18-22 months of age in a dose-escalation study starting at 0.5mg/kg of melatonin increasing to 5mg/kg if smaller doses are well tolerated, again as an adjunctive therapy to TH (NCT02621944).

Other neuroprotective strategies for patients with NE are being investigated, including erythropoietin (Epo), allopurinol, and magnesium sulfate (MgSO<sub>4</sub>). Phase II trials of some therapies such as Epo have demonstrated safety and neuroprotective benefits including improved MRI injury score and motor outcomes at 12 months in the treatment of NE (Wu, 2016). However, the certainty in these results remains low due to confounding and phase III trials are ongoing. The efficacy and safety of each individual intervention must be established first, but there is likely to be a role of many therapeutic interventions that target different phases of injury in NE and studies on pre-clinical models of NE are investigating these possibilities (Robertson, 2020).

One major challenge for researching therapeutic interventions for NE is that the condition is much more prevalent in low-resource settings and the only neuroprotective strategy, TH, is not widely available or safe in these settings (Robertson 2008). Therefore, adjunctive therapies to TH and single interventions must be examined separately, as TH may modify the effects of any other intervention including melatonin treatment. We completed a subgroup analysis of the effects of melatonin monotherapy or melatonin as an adjunctive therapy to TH on the effects on mortality. Melatonin resulted in reduced mortality in the monotherapy group, but not in the adjunctive treatment group. There was very low certainty evidence for these conclusions but melatonin treatment may be a promising intervention when TH is not available, and this requires further investigation.

A limitation of the assessment of any neuroprotection intervention in the treatment of NE is the variation in outcome measurement. This limitation is reflected in this Cochrane review, as all studies measured different outcomes, or the same outcome at different timepoints. This results in challenges in comparing study outcomes and the inability to complete meta-analyses. A core outcome set which aims to standardise outcomes measured and reported in trials of interventions for the treatment of NE is currently being formulated and should be implemented in future trials (Quirke, 2020).

#### Quality of the evidence

We judged all included studies to be unclear risk of bias. For this reason and due to concerns regarding imprecision and inconsistency in the result estimates, the overall certainty of the evidence from this Cochrane review is very low for each outcome presented in the Summary of Findings.

We applied the GRADE approach to assess the certainty of the evidence for this review. We downgraded the certainty of evidence for the outcomes mortality and NDD, or NDD alone, by two levels due to very serious imprecision as the study included a very small sample size and only the results of significance testing were reported, and by one level due to the study limitations as the only included study was judged to be at unclear risk of bias. We downgraded the certainty of evidence for the outcome of mortality by one level due to each of risk of bias in included studies, imprecision, and inconsistency in study findings. The certainty of evidence for the outcome abnormalities on MRI brain were downgraded by two

levels due to very serious imprecision as there were very few events from two small-sample RCTs and the confidence interval included appreciable benefits and harm, and by one level due to unclear risk of bias in both included studies.

#### Potential biases in the review process

We adhered to standard methodological procedures expected by Cochrane Neonatal, and followed the criteria and methodology outlined in the Methods section for this review. To minimise the risk of bias and errors in this review two authors were employed to independently screen records, extract data, assess the risk of bias in included studies, and evaluate the certainty of the evidence. The search strategy is outlined above and no restrictions by language or publication type were applied. As the number of records yielded by the search strategy were low, we made significant efforts to identify further relevant studies by completing an extensive search of the grey literature and contacting key experts in this research area. Although the possibility remains that there may be relevant unpublished studies that we have not included, it is unlikely that through our efforts we failed to identify any relevant study. Another recent systematic review did not include any other studies suitable for our review. Unfortunately, as the number of included studies is low, we cannot evaluate publication bias in this review using a funnel plot.

#### Agreements and disagreements with other studies or reviews

We identified one recent systematic review that evaluated the evidence for the use of melatonin treatment for patients with NE. Ahmad included RCTs of melatonin compared

to placebo, or melatonin as an adjuvant therapy to TH compared to melatonin alone, or melatonin plus Epo or MgSO<sub>4</sub> compared to a control arm. The primary outcomes for the systematic review were neurodevelopmental impairment at 18-24 months and death before discharge. The review included five studies, including four of the same studies included in this Cochrane review. Ahmad included a further study which assessed the effect of melatonin plus MgSO<sub>4</sub> compared to melatonin alone in participants with HIE (El Farargy 2019). We did not include this RCT in this Cochrane review as we did not intend to examine melatonin as an adjunctive therapy to interventions outside of standard treatment, including TH, and both groups in the RCT received melatonin treatment. Another major difference with this systematic review was the risk of bias assessment. In this Cochrane review we judged the blinding of participants and personnel to be low risk of bias in three of the four studies. We judged the blinding process either to be adequate or assessment of the outcome, mortality, to be unlikely to be influenced by the lack of blinding. We also judged the outcome assessment in the RCT trial by Ahmad to be at low risk of bias as the only outcome, mortality, is unlikely to be influenced by the lack of blinding. For this Cochrane review we did not consider the absence of treatment with TH a risk of bias as we intended to review the evidence of the effect of melatonin treatment as a single agent and as an adjunctive therapy to TH. The authors of the Ahmad review concluded that the RCT by Jerez-Calero was the only trial of melatonin to provide long-term neurodevelopmental follow-up, and that participants in the melatonin treatment group had better cognitive ability on Bayley-III at 18 months compared to the placebo group. They also concluded that there was no evidence of a reduction in mortality in participants treated with melatonin as

an adjuvant therapy to TH compared to TH alone, but the certainty of evidence for this conclusion was very low. The major difference in conclusion is that the authors of the Ahmad review did not complete a meta-analysis of RCTs that compared melatonin treatment alone without TH, as they judged both relevant included studies to be of very low quality.

We also identified 1 small study that evaluated the pharmacokinetics (PK) of melatonin in patients undergoing TH (Balduini, 2019). This study enrolled 5 participants diagnosed with moderate-severe NE within the first 12 hours of life. Participants received an enteral infusion of melatonin via orogastric tube infused over four hours at a dose of 0.5mg/kg, the lowest dose approved by the FDA for the study. During the period of melatonin administration participants vital signs, systemic and cerebral oxygenation, and abdominal exams were monitored. Serial blood samples were taken at regular intervals to evaluate the PK of melatonin in patients undergoing TH. Melatonin administration was well tolerated in this study, although the melatonin dose is lower than the RCTs included in this Cochrane review. The authors concluded that TH did not affect melatonin PK, that enteral doses of melatonin should not exceed 1-5mg/kg, however IV administration is the preferred route of melatonin administration as it bypasses absorption by the gastric mucosa thereby achieving higher and more consistent blood melatonin concentrations. The study authors do not comment on the appropriate dose of melatonin when administered IV.



We identified RCTs in other neonatal populations that examined the effect of melatonin treatment on participant outcomes. Like NE, the pathophysiology of these diagnoses involves oxidative stress as a prominent feature; sepsis (N = 20) (Gitto 2001), RDS (N = 74) (Gitto 2004), and prematurity (N = 110) (Gitto 2005). Dosage of melatonin in these studies ranged from 20mg in two 10mg doses, to 100mg/kg, in ten 10mg/kg doses. All studies reported improved outcome in the melatonin treatment groups. Most studies measured clinical outcomes including a reduction in chronic lung disease, necrotizing enterocolitis, and retinopathy of prematurity, however some studies only measured the effect of melatonin on inflammatory markers. A retrospective review of these studies (N = 85) noted that no adverse event had been observed in a participant treated with melatonin in any of the RCTs (Aversa, 2012).

The promise of melatonin as a neuroprotective strategy has been elucidated in many recent reviews that highlight the anti-inflammatory, anti-oxidant and anti-apoptotic properties that may target the pathophysiology underlying brain injury in NE (Cardinali DP 2019, Tarocco 2019, Pang 2021). Although there is a paucity of clinical evidence for the use of melatonin in human trials, there is significant evidence from pre-clinical studies. Melatonin treatment has demonstrated improved neuroprotection in piglet, sheep, and lamb models of NE (D'angelo 2020). Melatonin treatment resulted in reduced markers of neuroinflammation, increased oligodendrocyte cell numbers, and reduced markers of neuronal cell death. It is 20 years since the first in-vivo studies of melatonin treatment for neonatal participants were conducted and the lack of progress on the evaluation of

melatonin as an adjunctive therapy to TH since then is outlined in a recent editorial (Pang, 2021). Some reasons for this delay are discussed including the highly lipophilic nature of melatonin necessitating the use of ethanol as an excipient and the difficulties developing a safe IV formulation.

The evidence from our review provides low certainty evidence that melatonin as an adjunctive therapy to TH provides neuroprotection. This requires urgent evaluation by large phase III RCTs which evaluate neurodevelopmental outcomes at over 18 months of age.

## Conclusion

### Implications for practice

We remain uncertain whether melatonin treatment reduces the incidence of the combined outcome of mortality and adverse long-term neurodevelopmental disability. Only one pilot study reported this outcome and the sample size was insufficient to draw definitive conclusions. All included studies reported improved outcomes with melatonin treatment compared to placebo or standard treatment, whether or not that included therapeutic hypothermia. Those outcomes included reduced incidence of mortality and neurodevelopmental delay. Despite promising early results, there is currently insufficient evidence to recommend the routine use of melatonin treatment for patients with NE.

## Implications for research

The promising results of melatonin urgently require further evaluation. Despite the routine use of TH in high resource settings, NE remains one of the leading causes of death and long-term neurodisability in term newborns. Furthermore, recent trials have demonstrated the lack of safety and effectiveness of TH in low- and middle-income countries. There is an urgent need for treatment options as adjuncts to TH and as single interventions. The promising early results for melatonin treatment mean there is an urgent need for further evaluation of this treatment option to establish whether its introduction as a routine treatment for patients with NE would lead to improved outcomes.

## Chapter 6: Effects of melatonin treatment on inflammatory cytokines in infants with NE

### Abstract

**Background:** Despite routine use of therapeutic hypothermia (TH) for patients with neonatal encephalopathy (NE), the associated mortality or severe disability in survivors remains close to 50%. There is an urgent need for further neuroprotection strategies. Adverse outcome in NE is associated with dysregulated inflammation. Melatonin is a potent anti-inflammatory, anti-oxidant and anti-apoptotic agent. Melatonin has been demonstrated to be a safe and effective therapy in animal models of NE and appears to be a promising therapy in early human trials.

**Methods:** Infants diagnosed with moderate-severe NE, who were undergoing TH, were prospectively recruited (n=36) and concurrent controls (n=18). Samples were collected during the first week of life. Whole blood samples were treated with melatonin, endotoxin, (lipopolysaccharide, LPS) or both. The effects of melatonin treatment and endotoxin stimulation on cytokine production were examined by multi-plex ELISA. The 14 cytokines examined were EPO, GM-CSF, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1RA, IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-18, TNF- $\alpha$ , TNF- $\beta$ , and VEGF.

**Results:** Infants with NE a significant reduction in cytokines GM-CSF and IFN- $\gamma$  and a significant increase in cytokines IL-1RA, IL-8, TNF- $\alpha$ , and VEGF following melatonin

treatment. In endotoxin stimulated samples, melatonin treatment resulted in a significant increase in EPO, GM-CSF, IFN- $\gamma$ , IL-1RA, IL-2, IL-6, IL-8, IL-10, IL-18, TNF- $\alpha$ , TNF- $\beta$ , and VEGF. Compared to controls, the IFN- $\gamma$ , IL-10, IL-18, and VEGF response to melatonin treatment was significantly lower in of infants with NE. Infants with NE had a significantly greater EPO, IL-1RA, IL-6, IL-8, TNF- $\alpha$  and significantly lower in IL-2 with melatonin and LPS in infants with NE compared to controls.

**Discussion:** Melatonin is a potent immunomodulating agent in infants with NE undergoing therapeutic hypothermia. There were significant differences in cytokine response to melatonin treatment in unstimulated and endotoxin stimulated groups in infants with NE. Melatonin treatment affected several cytokines differently in infants undergoing TH for NE compared to controls at baseline and in response to LPS.

## Introduction

Neonatal encephalopathy (NE) is the fifth most common cause of death under five globally [449], accounting for an estimated 287000 deaths annually [11]. Dysregulated inflammation plays a major pathophysiological role in NE [105, 450], exacerbates brain injury [103], and leads to adverse outcomes [107]. Cytokines have been implicated in the pathogenesis of NE [118, 168]. Dysregulation of pro- and anti-inflammatory cytokine production is associated with increased brain injury on MRI [104] and increased mortality [101]. Persistent cytokine dysregulation production continues beyond the neonatal period into childhood following NE [23].

Cytokines are polypeptides secreted by leukocytes and other immune cells that act primarily on haematopoietic cells to regulate immune and inflammatory reactions [451]. The definition is broad and there is some overlap with the classification of hormones, although cytokines are much more potent and are produced by many cell types as opposed to a single cell or tissue [97]. There are many different types of cytokines, and they are frequently classified into families according to the type of receptor to which they bind or by their function [452]. Dysregulation of several cytokine families has been demonstrated in NE [100-102, 104, 450]. The interleukin (IL)-1 receptor family cytokines IL-1 $\beta$  and IL-18 which are potent pro-inflammatory cytokines, and their antagonist IL-1RA, type 1 (haematopoietin) receptor family cytokines of which there are important pro-and anti-inflammatory cytokines, type 2 (interferon) receptor family & tumour necrosis factor family cytokines which include major immune-regulating cytokines, and CXC chemokine family &

vascular endothelial growth factor cytokines which promote inflammation and angiogenesis. There is significant interaction and interdependence between cytokine families. Diurnal variation in cytokine production has also been demonstrated with peak production of pro-inflammatory cytokines occurring at night and in the early morning [453]. Dynamic changes following therapeutic interventions have correlated changes in cytokine production to improved outcomes in NE [107].

Melatonin is an endogenous polypeptide with potent immune-modulating properties [108]. It has immune-stimulating properties enhance resistance to pathogens in infection [454], and anti-inflammatory properties including prevention of the production of pro-inflammatory cytokines such as IL-1 $\beta$  by preventing inflammasome formation [455], prevention of reactive oxygen species release (ROS) [182], scavenging of ROS [456], and protection of mitochondrial function [457]. Melatonin inhibits activation of nuclear factor kappa B (NF $\kappa$ B) [458], altering the production of several range of cytokines [421]. For these reasons, melatonin has been proposed as an immune-modulation treatment for infants with NE [181].

## Hypothesis

We hypothesised that melatonin treatment would alter cytokines in infants with NE and controls, and that the effects of melatonin treatment would be influenced by the presence of NE, endotoxin treatment, and time-of-day of sampling.

## Aims

To examine the effects of melatonin treatment in vehicle and endotoxin-treated samples on a panel of cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-2, IL-6, IL-8, IL-10, IL-18, IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , EPO and VEGF), in both infants with NE and controls.



## Methods

### Study Population

Infants with neonatal encephalopathy (NE) and controls were recruited from three Dublin maternity hospitals. There are over 8000 deliveries annually in each hospital and each has a tertiary neonatal intensive care unit which is a national centre for therapeutic hypothermia. Infants were included if they were diagnosed with NE, and met previously published inclusion criteria [105, 450, 459]. Infants with NE were classified by severity of neurological dysfunction according to Sarnat staging [288]. Infants were excluded if they were born prematurely at <35 weeks GA, had major congenital or chromosomal abnormalities, or if there was evidence of maternal substance misuse. Healthy term infants were recruited as controls if they were undergoing phlebotomy from day 1–7 of life, but infants were excluded if there was suspicion for sepsis or if they were receiving phototherapy for hyperbilirubinaemia.

### Sampling and cytokine analysis

Blood samples were collected on all patients within the first 7 days of life in collection bottles with sodium citrate solution (maximum volume 1.4ml) and transferred to the laboratory to ensure processing commenced within one hour. Samples were collected during routine patient phlebotomy from umbilical or peripheral arterial lines when available, or otherwise from peripheral venous samples. 100µl samples of whole blood were used for each treatment group. Samples were treated with phosphate-buffered solution (PBS) with 0.15% ethanol, LPS (E.coli 0111:B4: SIGMA Life Science, Wicklow, Ireland) (10

ng/mL), and melatonin (SIGMA Life Science, Wicklow, Ireland) (1.2µL of 42mM in 0.15% ethanol solution) alone and in combination and incubated for 1 hour at 37°C. Samples were then centrifuged (10 minutes at 4°C at 1500rpm) following which the plasma was collected and stored at -80°C for later batch processing.

Cytokines were analysed by sandwich enzyme linked immunosorbent assay (ELISA) multiplex cytokine array using a 10-spot and 5-spot plasma 96-well plates customised for our study and selected cytokines by MesoScale Discovery ([www.meso-scale.com](http://www.meso-scale.com)). Biotinylated capture antibodies, specific to the cytokines of interest in this study, were attached to U-PLEX linkers which then self-assemble onto unique spots in the U-PLEX plate. For this experiment 200µL of each biotinylated capture antibody was coupled to 300µL of a unique linker, a different linker used for each antibody. Each solution was vortexed, incubated for 30 minutes 200µL of Stop solution added, and incubated for a further 30 minutes. For both multi-array plates, 600µL of each U-PLEX coupled antibody solution were combined, resulting in a 1X multiplex coating solution. 50µL of the multiplex coating solution was added to each well of the 96-well plates. Plates were then incubated with shaking and washed. Calibrator standard and detection antibody solution were prepared per the manufacturer's instructions. 25µL of Diluent 43 was added to each well, all sides of the plate were tapped gently, and 25µL of the prepared calibrator standard solution was added to the calibrator wells. 25µL of collected supernatant samples were then added to the plates. Analytes (cytokines of interest) within these samples bound to the capture antibodies-linker complexes. Detection antibodies (50µL) conjugated with electrochemiluminescent labels were

then added to the plates and incubated for one hour, which further bound to the analytes to form the sandwich immunoassay. Once the sandwich was complete, 150  $\mu$ l of Read Buffer T was added to each well and the plates were analysed on the Sector Imager and validated. The volume of detection antibody increases in proportion to the concentration of analyte present. Detection antibodies fluoresce when a current is applied to the plate, and the volume of analyte present is represented by the intensity of light produced. The 13 cytokines analysed were erythropoietin (Epo), granulocyte and Granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-2, IL-6, IL-8, IL-10, IL-18, interferon (IFN)- $\gamma$ , tumour necrosis factor (TNF)- $\alpha$ , TNF- $\beta$ , and vascular endothelial growth factor (VEGF). Results were displayed in pg/ml [177].

## Statistics

The presumption of normal distribution for the collected data was examined by visual assessment of histograms and by the Shapiro-Wilk test, a formal test to assess whether or not a sample fits a normal distribution. If the presumption was violated, outliers were further examined for clinical or methodological variability. If outliers could not be explained all data was retained in the analysis and a log transformation was applied to the data or non-parametric tests were used where appropriate. If the presumption of normality of data distribution was violated, differences in cytokine concentrations between treatment groups within the sample population cohort were compared using the Wilcoxon signed rank test for paired samples, for the groups vehicle vs melatonin, vehicle vs LPS, and LPS vs LPS & melatonin. Differences in cytokine concentrations between treatment groups within the

sample timing group (day or night) were also compared using the Wilcoxon signed rank test for paired samples. Differences in cytokine concentrations between NE and control groups for the same treatment group were compared using the Mann-Whitney U test. Differences in cytokine concentrations between day and night sampling groups for the same treatment group were also compared using the Mann-Whitney U test. Repeat samples were obtained for some infants with NE, however only the first patient sample obtained was included for this analysis. All these tests were 2-tailed, and the level of significance was set at  $p < 0.05$ .

The interaction between population cohort and treatment group was examined by 2-way mixed analysis of variance (ANOVA) for log-transformed data to assess whether the interaction between the presence of NE and treatment group affected the change in cytokines. The interaction between timing of sampling and treatment group was examined by 2-way mixed ANOVA for log-transformed data to assess whether the interaction between the timing of sampling and treatment group affected the change in cytokines. The effect size was measured by the partial eta squared ( $\eta^2$ ), and the level of significance was set at  $p < 0.05$ .

### Ethical approval

This study was approved by the research ethics committee approved this study in each of the three included maternity hospitals, the Coombe Women's and Infant's University Hospital, the Rotunda Hospital, and the National Maternity Hospital (Appendix I). Written informed consent was required from the parents or guardians, who were provided

with verbal and a written patient information leaflet, for each included infant prior to enrolment in the research study (Appendix II & III).

## Results

### Infant characteristics

36 patients with NE and 18 healthy controls were recruited to this study. All infants with NE were treated with therapeutic hypothermia. The median gestational age (GA) at birth for infants with NE was 38 weeks compared to mean GA of 40+3 weeks at birth for healthy controls. The median birthweight for those with NE was 3200gms, compared to 3900gms for healthy controls. The same proportion of participants were female in both groups, at 33%. A higher proportion of infants with NE (50%) were born by lower segment caesarean section compared to healthy controls (22%). Compared to healthy controls, infants with NE had lower Apgar scores at 1 minute, median 9 and 1 respectively, and at 5 minutes, median 9 and 4 respectively. The majority of infants with NE were intubated (92%) however only a small proportion required resuscitation with cardiopulmonary resuscitation (8%). All infants with NE underwent therapeutic hypothermia (TH) and the majority of patients had seizures prior to discharge from hospital (70%). Of the 33 patients that survived to discharge, 20 had normal MRI brain (60%).

	NE (n = 36)	Control (n = 18)
Gestational Age in weeks (median, IQR)	38 (38 -40)	40+3 (38 – 41)
Birthweight in grams (median, IQR)	3200 (2820 – 3630)	3900 (3400 – 4130)
Gender (female %)	12 (33)	6 (33)
LSCS (%)	18 (50)	4 (22)
Apgar 1 minute (median, IQR)	1 (1 – 3.5)	9 (8-9)
Apgar 5 minute (median, IQR)	4 (2 - 6)	9 (9)
CPR (%)	3 (8)	N/A
Intubated (%)	33 (92)	N/A
Cord arterial pH	7.09 (6.9 – 7.2)	N/A
Cord arterial BE	8.9 (7.5 – 16.7)	N/A
Therapeutic hypothermia (%)	36 (100)	N/A
Seizures (%)	25 (70)	N/A
MRI abnormal (%)	20 (60)	N/A

**Table 18 Participant characteristics for those born with neonatal encephalopathy (NE) and healthy controls.** Results are presented as median and interquartile range, or as percentages. IQR – interquartile range; LSCS – lower segment caesarean section; BE – base excess.

## Effect of melatonin treatment on cytokine levels in infants with NE and controls

Statistical results of the distribution of cytokine concentration in vehicle and melatonin treated samples from infants with NE and controls were not normally distributed (Appendix VI) This was confirmed by Shapiro-Wilk test, with p values  $<0.05$  considered evidence against the null hypothesis of a normal distribution (Appendix VI).



Cytokine	Veh NE	Mel NE	p value	Veh Control	Mel Control	p value
IL-1 $\beta$	0.80 (0.51 – 1.11)	0.84 (0.41 – 1.41)	0.812	0.61 (0.33 – 1.01)	0.53 (0.32 – 1.15)	0.542
IL-18	368.25 (258.13 – 596.93)	404.72 (338.50 – 554.38)	0.120	235.89 (155.68 – 287.09)	289.26 (203.38 – 370.16)	<b>&lt;0.001</b>
IL-1RA	2687.10 (1393.84 – 5276.17)	3218.20 (1427.54 – 6835.36)	<b>&lt;0.001</b>	717.06 (433.22–1160.10)	1057.60 (764.64 – 1448.34)	<b>0.001</b>
IL-2	0.18 (0.00 – 0.43)	0.00 (0.00 – 0.25)	<b>0.031</b>	0.32 (0.105 – 0.645)	0.40 (0.02 - 0.77)	0.647
IL-6	15.79 (7.82 - 30.71)	15.83 (10.00 – 33.31)	0.105	3.03 (1.51 – 6.72)	5.79 (2.15 – 8.80)	<b>0.001</b>
EPO	186.28 (57.31 – 356.79)	177.56 (54.44 – 352.51)	0.451	26.47 (17.81 – 41.14)	32.49 (23.03 – 52.10)	<b>0.002</b>
GM-CSF	0.16 (0.08 – 0.24)	0.07 (0.00 – 0.12)	<b>0.004</b>	0.29 (0.10 – 0.33)	0.19 (0.07 – 0.37)	0.052
IFN- $\gamma$	5.06 (2.78 – 10.28)	2.41 (0.01 – 6.36)	<b>0.003</b>	14.43 (7.96 – 45.86)	15.77 (8.04 – 57.22)	<b>0.028</b>
IL-10	0.91 (0.71 – 1.57)	0.98 (0.55 – 1.50)	0.121	0.85 (0.54 – 1.08)	1.09 (0.77 – 1.59)	0.055
TNF- $\alpha$	8.61 (5.84 – 10.84)	9.27 (6.49 – 17.56)	<b>0.008</b>	6.63 (3.63 – 8.65)	7.71 (5.71 0 10.73)	0.074
TNF- $\beta$	0.48 (0.15 – 0.83)	0.53 (0.00 – 1.45)	0.282	0.30 (0.16 – 0.46)	0.25 (0.11 – 0.64)	0.298
IL-8	68.62 (43.44 – 110.76)	88.5 (49.75 – 148.86)	<b>0.006</b>	29.82 (21.08 – 61.09)	36.82 (27.19 – 142.29)	<b>0.016</b>
VEGF	66.96 (45.13 – 94.38)	87.16 (58.50 – 118.23)	<b>&lt;0.001</b>	82.27 (49.67 – 113.87)	120.38 (71.60 – 201.28)	<b>0.001</b>

**Table 19 Cytokines in infants with NE and controls: Results (median and interquartile range) for cytokines in pg/ml. Differences between vehicle and melatonin treated samples for the same patient groups were examined by Wilcoxon rank sum test for paired samples. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.**

Cytokine	Veh NE	Veh Control	p value	Mel NE	Mel Control	p value
IL-1 $\beta$	0.80 (0.51 – 1.11)	0.61 (0.33 – 1.01)	0.137	0.84 (0.41 – 1.41)	0.53 (0.32 – 1.15)	0.320
IL-18	368.25 (258.13 – 596.93)	235.89 (155.68 – 287.09)	<b>&lt;0.001</b>	404.72 (338.50 – 554.38)	289.26 (203.38 – 370.16)	<b>0.002</b>
IL-1RA	2687.10 (1393.84 – 5276.17)	717.06 (433.22-1160.10)	<b>&lt;0.001</b>	3218.20 (1427.54 – 6835.36)	1057.60 (764.64 – 1448.34)	<b>&lt;0.001</b>
IL-2	0.18 (0.00 – 0.43)	0.32 (0.105 – 0.645)	0.333	0.00 (0.00 – 0.25)	0.40 (0.02 – 0.77)	<b>0.008</b>
IL-6	15.79 (7.82 - 30.71)	3.03 (1.51 – 6.72)	<b>&lt;0.001</b>	15.83 (10.00 – 33.31)	5.79 (2.15 – 8.80)	<b>&lt;0.001</b>
EPO	186.28 (57.31 – 356.79)	26.47 (17.81 - 41.14)	<b>&lt;0.001</b>	177.56 (54.44 – 352.51)	32.49 (23.03 – 52.10)	<b>&lt;0.001</b>
GM-CSF	0.16 (0.08 – 0.24)	0.29 (0.10 – 0.33)	0.071	0.07 (0.00 – 0.12)	0.19 (0.07 – 0.37)	<b>0.005</b>
IFN- $\gamma$	5.06 (2.78 – 10.28)	14.43 (7.96 – 45.86)	<b>0.003</b>	2.41 (0.01 – 6.36)	15.77 (8.04 – 57.22)	<b>&lt;0.001</b>
IL-10	0.91 (0.71 – 1.57)	0.85 (0.54 – 1.08)	0.384	0.98 (0.55 – 1.50)	1.09 (0.77 – 1.59)	0.419
TNF- $\alpha$	8.61 (5.84 – 10.84)	6.63 (3.63 – 8.65)	<b>0.032</b>	9.27 (6.49 – 17.56)	7.71 (5.71 – 10.73)	0.159
TNF- $\beta$	0.48 (0.15 – 0.83)	0.30 (0.16 – 0.46)	<b>0.037</b>	0.53 (0.00 – 1.45)	0.25 (0.11 – 0.64)	0.621
IL-8	68.62 (43.44 – 110.76)	29.82 (21.08 – 61.09)	<b>0.002</b>	88.5 (49.75 – 148.86)	36.82 (27.19 – 142.29)	<b>0.019</b>
VEGF	66.96 (45.13 – 94.38)	82.27 (49.67 – 113.87)	0.243	87.16 (58.50 – 118.23)	120.38 (71.60 – 201.28)	0.071

**Table 20 Cytokines in infants with NE and controls: Results (median and interquartile range) for cytokines in pg/ml. Differences between vehicle and melatonin treated samples for between patient groups were examined by Mann-Whitney U test. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.**

Cytokine	Effect size ( $\eta^2$ )	p value
IL-1 $\beta$	0.030	0.216
IL-18	0.133	<b>0.008</b>
IL-1RA	0.059	0.081
IL-2	0.028	0.232
IL-6	0.103	<b>0.020</b>
EPO	0.154	<b>0.004</b>
GM-CSF	0.013	0.420
IFN $\gamma$	0.205	<b>0.001</b>
IL-10	0.130	<b>0.008</b>
TNF- $\alpha$	0.002	0.765
TNF- $\beta$	0.001	0.812
IL-8	0.052	0.102
VEGF	0.023	0.276

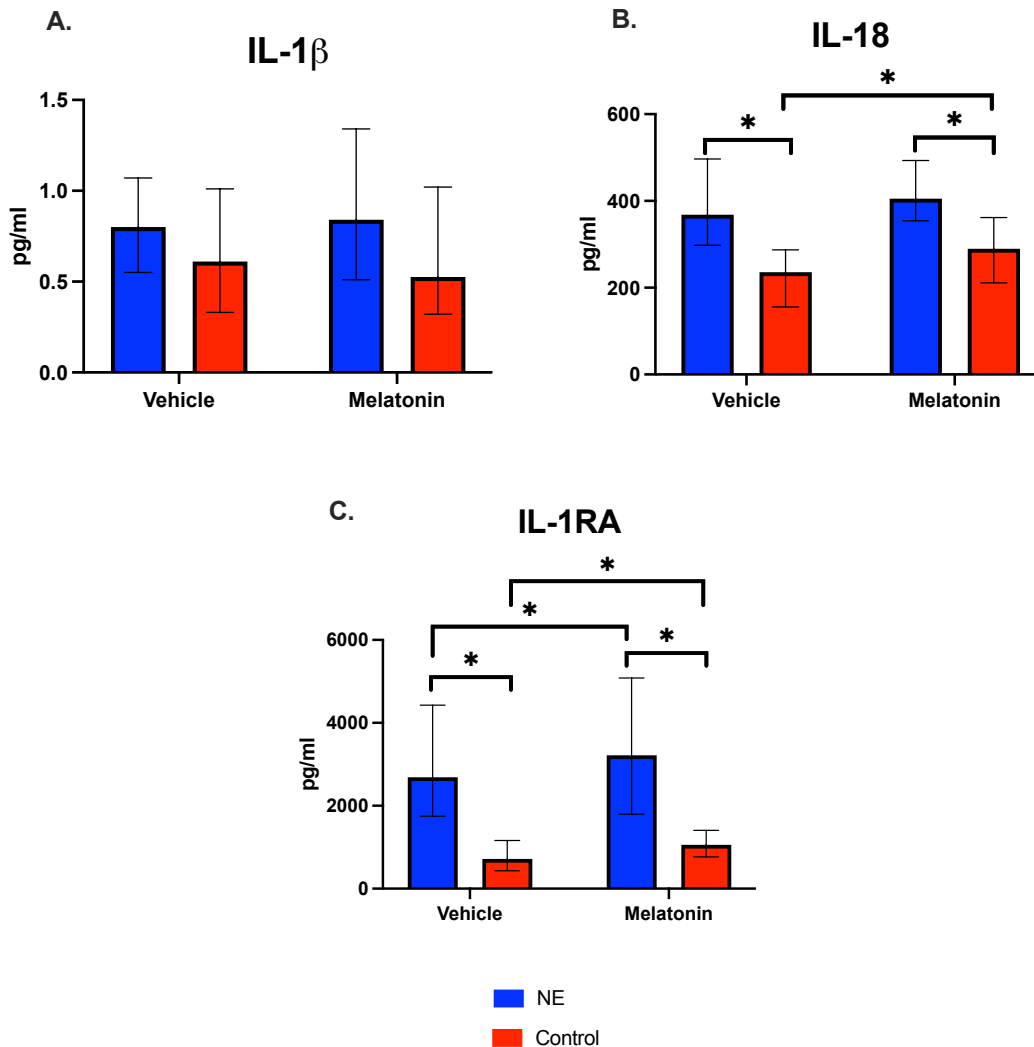
**Table 21** Effect size and significance testing results of mixed 2-way analysis of variance (ANOVA) for each cytokine comparing the effect of the presence of NE on the response to melatonin treatment.  $\eta^2$  - Eta squared, the effect size. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.

### IL-1 receptor family cytokines

No significant difference was found in IL-1 $\beta$  following melatonin in infants with NE or controls, nor was any significant difference found in between infants with NE and controls in IL-1 $\beta$  vehicle or melatonin-treated samples (Figure 65A). There was no interaction between the presence of NE and response to melatonin treatment ( $p = 0.216$ ) on IL-1 $\beta$  concentration.

IL-18 increased significantly following melatonin treatment in controls ( $p < 0.001$ ) but not in NE infants (Figure 65B). IL-18 was significantly higher in infants in NE compared to controls in both vehicle ( $p < 0.001$ ) and melatonin treated samples ( $p = 0.002$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.008$ ) on IL-18 concentration.

IL-1RA increased significantly following melatonin treatment in both infants with NE ( $p < 0.001$ ) and controls ( $p = 0.001$ ) (Figure 65C). IL-1RA was significantly higher in infants in NE in both vehicle ( $p < 0.001$ ) and melatonin treated samples ( $p < 0.001$ ). There was no interaction between the presence of NE and response to melatonin treatment ( $p = 0.081$ ) on IL-1RA concentration.



**Figure 65** The effect of melatonin treatment on IL-1 receptor cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in increased IL-18 in controls but not in infants with NE (B), and resulted increased IL-1RA in both infants with NE and controls (C). Melatonin treatment did not result in any changes in IL-1 $\beta$  (A). IL-18 was higher in both infants with NE and controls at baseline and following treatment with melatonin (B). IL-1RA was higher in both infants with NE and controls at baseline and following treatment with melatonin (B). IL – interleukin, RA – receptor antagonist.

\* indicates a significant difference between treatment or group, with a p value of <0.05

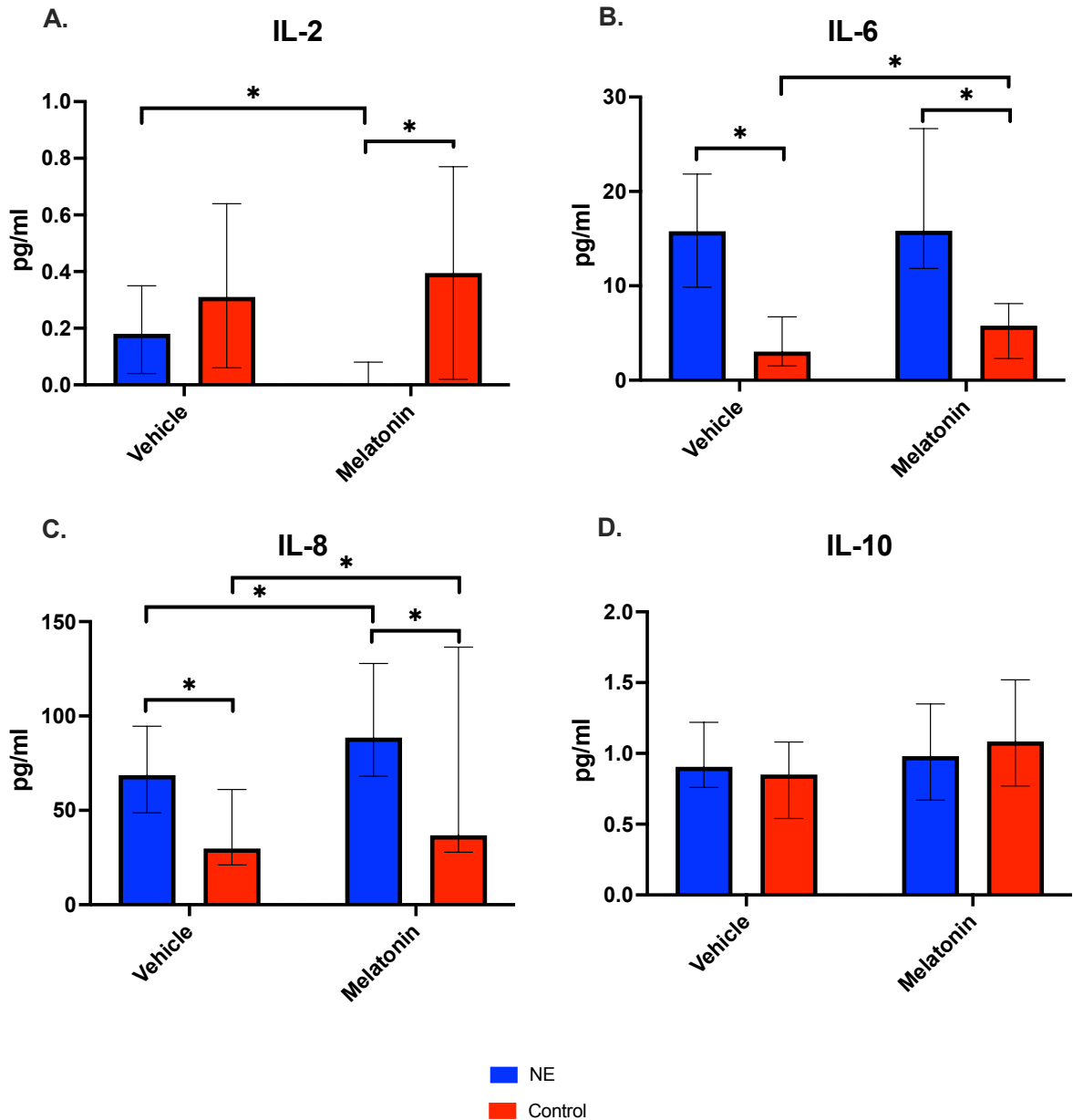
Type 1 (haematopoietin) receptor family cytokines

IL-2 decreased significantly following melatonin treatment in infants with NE ( $p = 0.031$ ) but not controls (Figure 66A). IL-2 was significantly lower in infants in NE in melatonin treated samples ( $p = 0.008$ ) compared to controls. There was no interaction between the presence of NE and response to melatonin treatment ( $p = 0.232$ ) on IL-2 concentration.

IL-6 increased significantly following melatonin treatment in controls ( $p = 0.001$ ) but not in infants with NE ( $p = 0.105$ ) (Figure 66B). IL-6 was significantly higher in infants with NE in both vehicle ( $p < 0.001$ ) and melatonin treated samples ( $p < 0.001$ ) compared to controls. There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.020$ ) on IL-6 concentration.

IL-8 increased significantly following melatonin treatment in both infants with NE ( $p = 0.006$ ) and controls ( $p = 0.016$ ) (Figure 66C). IL-8 was significantly higher in infants with NE in both vehicle ( $p = 0.002$ ) and melatonin treated samples ( $p = 0.019$ ) compared to controls. There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.102$ ) on IL-8 concentration.

No significant difference was found in IL-10 following melatonin in infants with NE or controls, nor was any significant difference found in between infants with NE and controls in IL-10 vehicle or melatonin-treated samples (Figure 66D). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.008$ ) on IL-10 concentration.



**Figure 66** The effect of melatonin treatment on type 1 (haematopoietin) receptor family cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in decreased IL-2 in infants with NE but not in controls (A), resulted increased IL-6 in controls but not in infants with NE(B), and resulted in increased IL-8 in both infants with NE and controls (C). There was no effect of melatonin treatment on IL-10 in either infants with NE or controls (D). IL-2 was higher in controls following melatonin treatment but there was no difference between groups at baseline (A). IL-6 and IL-8 were higher in infants with NE at both baseline and following melatonin treatment (B & C). IL – interleukin.

\* indicates a significant difference between treatment or group, with a  $p$  value of  $<0.05$

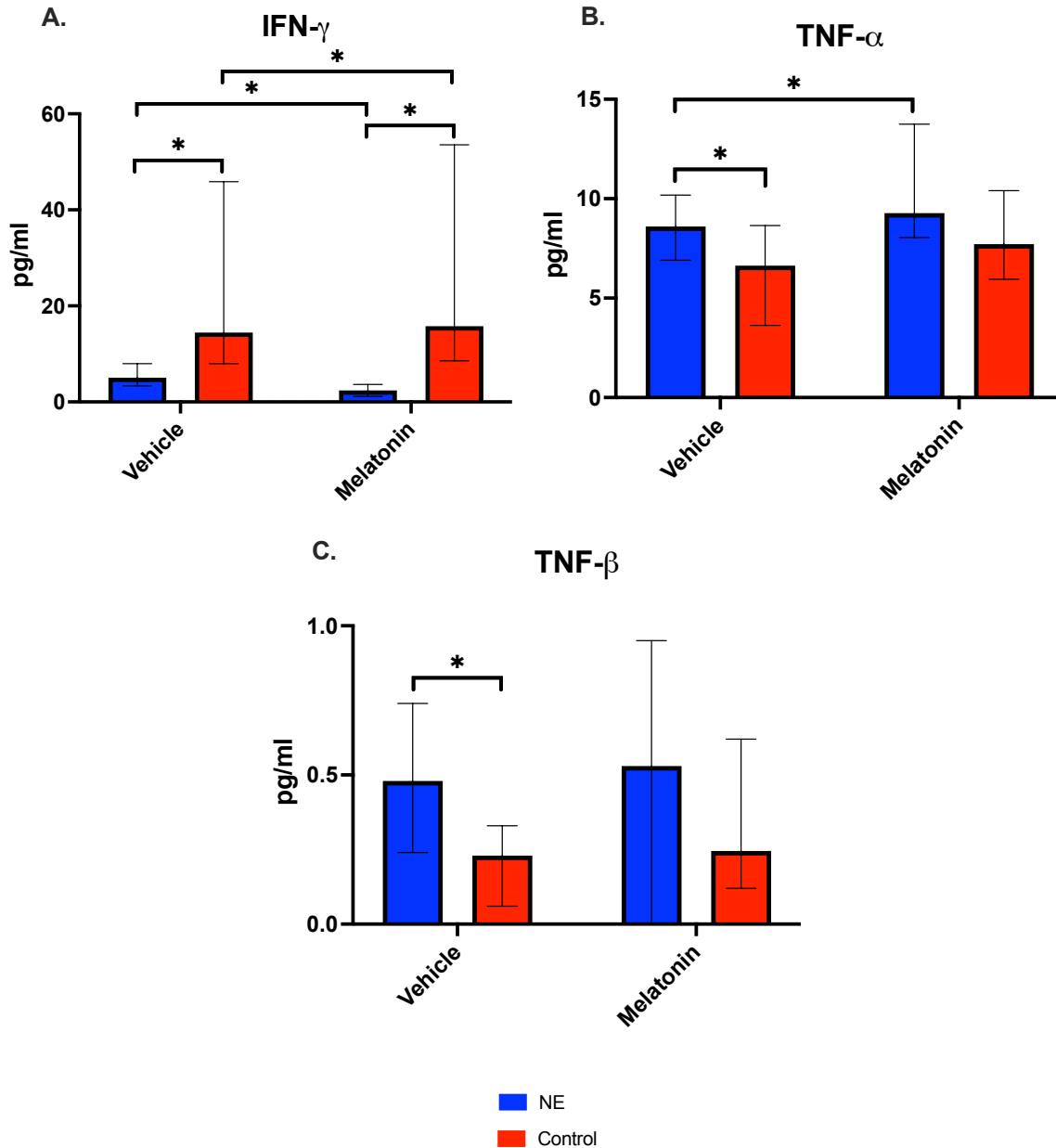
Type 2 (interferon) receptor family & tumour necrosis factor family cytokines

IFN- $\gamma$  decreased significantly following melatonin treatment in infants with NE ( $p = 0.003$ ) and increased significantly in controls ( $p = 0.028$ ) (Figure 67A). IFN- $\gamma$  was significantly lower in infants with NE in both vehicle ( $p = 0.003$ ) and melatonin treated samples ( $p < 0.001$ ) compared to controls. There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.001$ ) on IFN- $\gamma$  concentration.

TNF- $\alpha$  increased significantly following melatonin treatment in infants with NE ( $p = 0.008$ ) but not in controls (Figure 67B). TNF- $\alpha$  was significantly higher in infants with NE in vehicle ( $p = 0.032$ ) but not melatonin treated samples compared to controls. There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.765$ ) on TNF- $\alpha$  concentration.

TNF- $\beta$  was significantly higher in infants with NE in vehicle ( $p = 0.037$ ) but not melatonin treated samples compared to controls (Figure 67C). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.812$ ) on TNF- $\beta$  concentration.





**Figure 67** The effect of melatonin treatment on type 2 (interferon) receptor family & tumour necrosis factor family cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in decreased IFN- $\gamma$  in infants with NE and decreased IFN- $\gamma$  in controls (A). Melatonin treatment resulted in increased TNF- $\alpha$  in infants with NE but not in controls (B). Melatonin treatment did not result in any significant difference in TNF- $\beta$  in either infants with NE or controls (C). IFN- $\gamma$  was higher in controls at baseline and following melatonin treatment (A). TNF- $\alpha$  and TNF- $\beta$  were higher in infants with NE at baseline but not following treatment with melatonin (B & C). IFN – interferon; TNF – tumour necrosis factor.

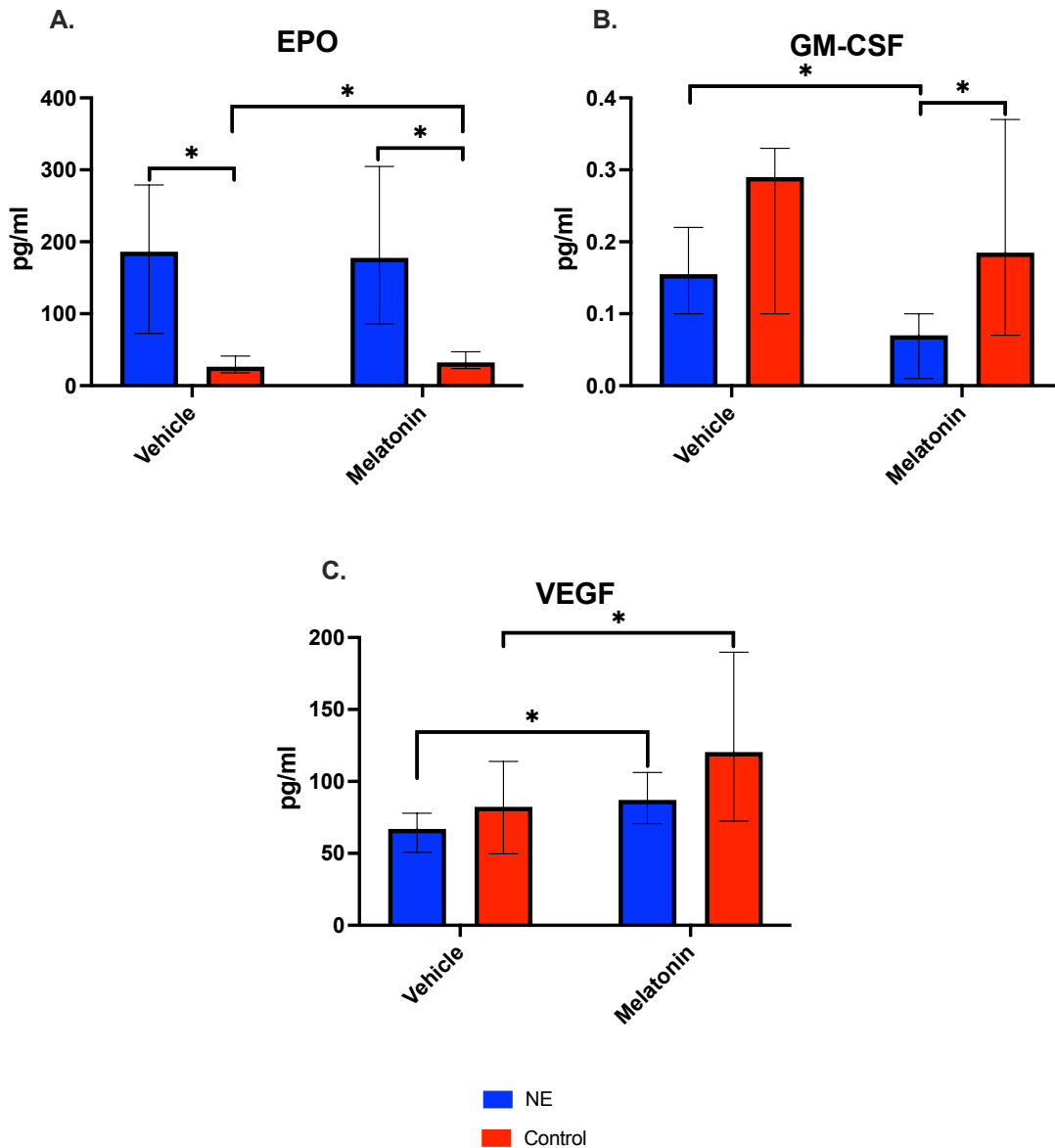
\* indicates a significant difference between treatment or group, with a p value of <0.05

### Haematopoiesis and angiogenesis stimulating cytokines

EPO increased significantly following melatonin treatment in controls ( $p = 0.002$ ) but not in infants with NE ( $p = 0.451$ ) (Figure 68A). EPO was significantly higher in infants with NE in both vehicle ( $p < 0.001$ ) and melatonin-treated samples ( $p < 0.001$ ) compared to controls. There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.004$ ) on EPO concentration.

GM-CSF decreased significantly following melatonin treatment in infants with NE ( $p = 0.004$ ) but not in controls ( $p = 0.052$ ) (Figure 68B). GM-CSF was significantly lower in infants with NE in melatonin-treated samples ( $p = 0.005$ ) and but not vehicle samples ( $p = 0.071$ ) compared to controls. There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.420$ ) on GM-CSF concentration.

VEGF increased significantly following melatonin treatment in both infants with NE ( $p < 0.001$ ) and controls ( $p = 0.001$ ) (Figure 68C). There was no significant difference in VEGF concentrations in infants with NE or controls in vehicle ( $p = 0.243$ ) or melatonin-treated samples ( $p = 0.071$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.276$ ) on VEGF concentration.



**Figure 68** The effect of melatonin treatment on haematopoiesis and angiogenesis stimulating cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in increased EPO in controls but not in infants with NE (A). Melatonin treatment resulted in decreased GM-CSF in infants with NE but not in controls (B). Melatonin treatment resulted in increased VEGF in both infants with NE and controls (C). EPO was higher in infants with NE both at baseline and following melatonin treatment (A). GM-CSF was higher controls following melatonin treatment but not at baseline (B). There was no difference in VEGF between groups either at baseline or following melatonin treatment (C). EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; VEGF – vascular endothelial growth factor.

\* indicates a significant difference between treatment or group, with a *p* value of <0.05

Effect of melatonin treatment on cytokines in endotoxin-stimulated samples from infants with NE and controls

Results of the distribution of cytokine concentration in endotoxin and endotoxin & melatonin treated samples from infants with NE and controls were analysed in the same manner as vehicle and melatonin sample results, by visual inspection of histogram graphs of the distribution of results and confirmation by Shapiro-Wilk tests. The results were not normally distributed, however all were included in the final analysis as any outliers could not be explained by clinical or methodological variability.

Cytokine	LPS NE	LPS+Mel NE	p value	LPS Control	LPS+Mel Control	p value
IL-1 $\beta$	0.96 (0.45 – 2.13)	1.65 (0.87 – 3.81)	0.169	0.85 (0.21 – 2.49)	0.85 (0.32 – 4.00)	0.136
IL-18	379.80 (294.67 – 496.81)	443.35 (348.70 – 631.61)	<0.001	252.75 (200.00 – 311.82)	275.87 (153.54 – 366.22)	0.356
IL-1RA	2630.34 (1379.73 – 5464.55)	3727.13 (2173.15 – 8334.16)	<0.001	1044.76 (554.80 – 1295.79)	964.83 (545.55 – 1410.22)	0.523
IL-2	0.00 (0.00 – 0.24)	0.10 (0.00 – 0.80)	0.064	0.17 (0.00 – 0.52)	0.40 (0.18 – 2.09)	<0.001
IL-6	15.25 (9.53 – 31.21)	20.61 (11.01 – 37.96)	0.005	5.82 (2.56 – 14.59)	7.56 (2.22 – 10.77)	0.687
IL-8	95.48 (53.75 – 162.61)	138.54 (75.54 – 236.03)	0.048	57.60 (39.01 – 332.23)	34.43 (17.70 – 284.18)	0.149
IL-10	0.71 (0.48 – 1.76)	1.18 (0.60 – 1.97)	0.004	0.87 (0.74 – 1.22)	1.01 (0.61 – 1.93)	0.256
IFN- $\gamma$	0.60 (0.00 – 3.72)	5.52 (1.79 – 9.62)	<0.001	10.86 (2.44 – 38.76)	16.74 (8.80 – 57.46)	0.044
TNF- $\alpha$	17.96 (12.09 – 35.87)	25.71 (16.92 – 58.92)	0.005	34.58 (17.69 – 82.60)	19.10 (8.23 – 72.56)	0.049
TNF- $\beta$	0.59 (0.24 – 1.11)	0.88 (0.40 – 1.96)	0.580	0.33 (0.08 – 0.84)	0.30 (0.17 – 0.77)	1.000
EPO	136.33 (59.10 – 369.40)	218.08 (78.86 – 406.99)	0.013	28.28 (20.52 – 43.13)	26.67 (21.01 – 43.54)	0.381
GM-CSF	0.00 (0.00 – 0.03)	0.11 (0.03 – 0.23)	<0.001	0.05 (0.00 – 0.19)	0.28 (0.07 – 0.43)	<0.001
VEGF	70.55 (47.39 – 108.84)	91.17 (51.40 – 148.57)	0.003	102.80 (65.97 – 168.75)	103.62 (37.25 – 192.26)	0.687

**Table 22 Cytokines in infants with NE and controls: Results (median and interquartile range) for cytokines in pg/ml. Differences between endotoxin-stimulated (LPS) and endotoxin & melatonin (LPS + Mel) treated samples for the same patient groups were examined by Wilcoxon rank sum test for paired samples. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.**

Cytokine	LPS NE	LPS Control	p value	LPS+Mel NE	LPS+Mel Control	p value
IL-1 $\beta$	0.96 (0.45 – 2.13)	0.85 (0.21 – 2.49)	1.000	1.65 (0.87 – 3.81)	0.85 (0.32 – 4.00)	0.190
IL-18	379.80 (294.67 – 496.81)	252.75 (200.00 – 311.82)	<b>&lt;0.001</b>	443.35 (348.70 – 631.61)	275.87 (153.54 – 366.22)	<b>&lt;0.001</b>
IL-1RA	2630.34 (1379.73 – 5464.55)	1044.76 (554.80 – 1295.79)	<b>0.002</b>	3727.13 (2173.15 – 8334.16)	964.83 (545.55 – 1410.22)	<b>&lt;0.001</b>
IL-2	0.00 (0.00 – 0.24)	0.17 (0.00 – 0.52)	0.563	0.10 (0.00 – 0.80)	0.40 (0.18 – 2.09)	<b>0.010</b>
IL-6	15.25 (9.53 – 31.21)	5.82 (2.56 – 14.59)	<b>0.011</b>	20.61 (11.01 – 37.96)	7.56 (2.22 – 10.77)	<b>&lt;0.001</b>
IL-8	95.48 (53.75 – 162.61)	57.60 (39.01 – 332.23)	0.647	138.54 (75.54 – 236.03)	34.43 (17.70 – 284.18)	<b>0.027</b>
IL-10	0.71 (0.48 – 1.76)	0.87 (0.74 – 1.22)	0.563	1.18 (0.60 – 1.97)	1.01 (0.61 – 1.93)	0.967
IFN- $\gamma$	0.60 (0.00 – 3.72)	10.86 (2.44 – 38.76)	<b>&lt;0.001</b>	5.52 (1.79 – 9.62)	16.74 (8.80 – 57.46)	<b>0.001</b>
TNF- $\alpha$	17.96 (12.09 – 35.87)	34.58 (17.69 – 82.60)	0.061	25.71 (16.92 – 58.92)	19.10 (8.23 – 72.56)	0.132
TNF- $\beta$	0.59 (0.24 – 1.11)	0.33 (0.08 – 0.84)	0.201	0.88 (0.40 – 1.96)	0.30 (0.17 – 0.77)	<b>0.005</b>
EPO	136.33 (59.10 – 369.40)	28.28 (20.52 – 43.13)	<b>&lt;0.001</b>	218.08 (78.86 – 406.99)	26.67 (21.01 – 43.54)	<b>&lt;0.001</b>
GM-CSF	0.00 (0.00 – 0.03)	0.05 (0.00 – 0.19)	<b>0.047</b>	0.11 (0.03 – 0.23)	0.28 (0.07 – 0.43)	<b>0.043</b>
VEGF	70.55 (47.39 – 108.84)	102.80 (65.97 – 168.75)	<b>0.033</b>	91.17 (51.40 – 148.57)	103.62 (37.25 – 192.26)	0.645

**Table 23 Cytokines in infants with NE and controls: Results (median and interquartile range) for cytokines in pg/ml.** Differences between endotoxin-stimulated (LPS) and endotoxin & melatonin treated samples for between patient groups were examined by Mann-Whitney U test. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.

Cytokine	Effect size ( $\eta^2$ )	p value
IL-1 $\beta$	0.032	0.216
IL-18	0.101	<b>0.026</b>
IL-1RA	0.470	0.135
IL-2	0.060	0.284
IL-6	0.008	0.536
IL-8	0.167	<b>0.004</b>
IL-10	0.001	0.819
IFN- $\gamma$	0.107	<b>0.048</b>
TNF- $\alpha$	0.246	<b>&lt;0.001</b>
TNF- $\beta$	0.065	0.121
EPO	0.031	0.225
GM-CSF	0.000	0.953
VEGF	0.083	<b>0.043</b>

*Table 24 Effect size and significance testing results of mixed 2-way analysis of variance (ANOVA) for each cytokine comparing the effect of the presence of NE on the response to melatonin treatment in endotoxin-stimulated samples.  $\eta^2$  - Eta squared, the effect size. The level of significance was set at  $p < 0.05$  and significant results are highlighted in bold. IL – interleukin; EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.*

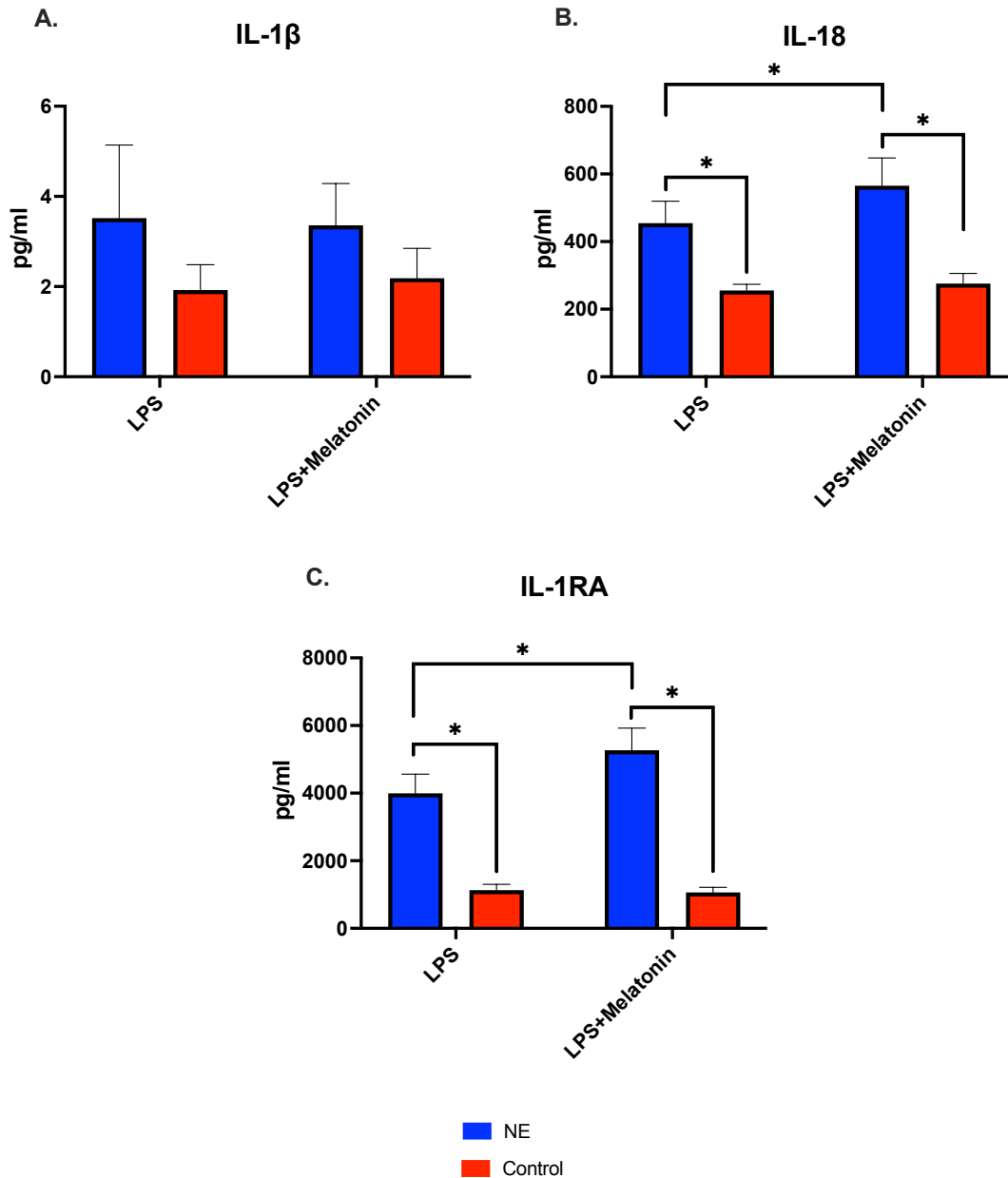
### IL-1 receptor family cytokines

No significant difference was found in endotoxin treated IL-1 $\beta$  following melatonin treatment in infants with NE or controls, nor was any significant difference found in between infants with NE and controls in endotoxin treated IL-1 $\beta$  vehicle or melatonin-treated samples (Figure 69A). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.850$ ) on endotoxin treated IL-1 $\beta$  concentration.

IL-18 increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p < 0.001$ ) but not in controls ( $p = 0.356$ ) (Figure 69B). IL-18 was significantly higher in infants in NE in both endotoxin treated ( $p < 0.001$ ) and endotoxin & melatonin treated samples ( $p < 0.001$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.026$ ) on endotoxin treated IL-18 concentration.

IL-1RA increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p < 0.001$ ) but not in controls ( $p = 0.523$ ) (Figure 69C). IL-1RA was significantly higher in infants in NE in both endotoxin treated ( $p = 0.002$ ) and endotoxin & melatonin treated samples ( $p < 0.001$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.135$ ) on endotoxin treated IL-1RA concentration.





**Figure 69** The effect of endotoxin-stimulation (LPS) and melatonin treatment on IL-1 receptor family cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in increased IL-18 and IL-1RA in infants with NE but not in controls (B & C). Melatonin treatment resulted no difference in IL-1 $\beta$  in either infants with NE or controls (A). IL-18 and IL-1RA were higher in both infants with NE and controls both at following endotoxin stimulating and following endotoxin & melatonin treatment (B & C). There was no difference between infants with NE or controls in IL-1 $\beta$  either following endotoxin stimulating nor following endotoxin & melatonin treatment (A). IL – interleukin.

\* indicates a significant difference between treatment or group, with a p value of <0.05

Type 1 (haematopoietin) receptor family cytokines

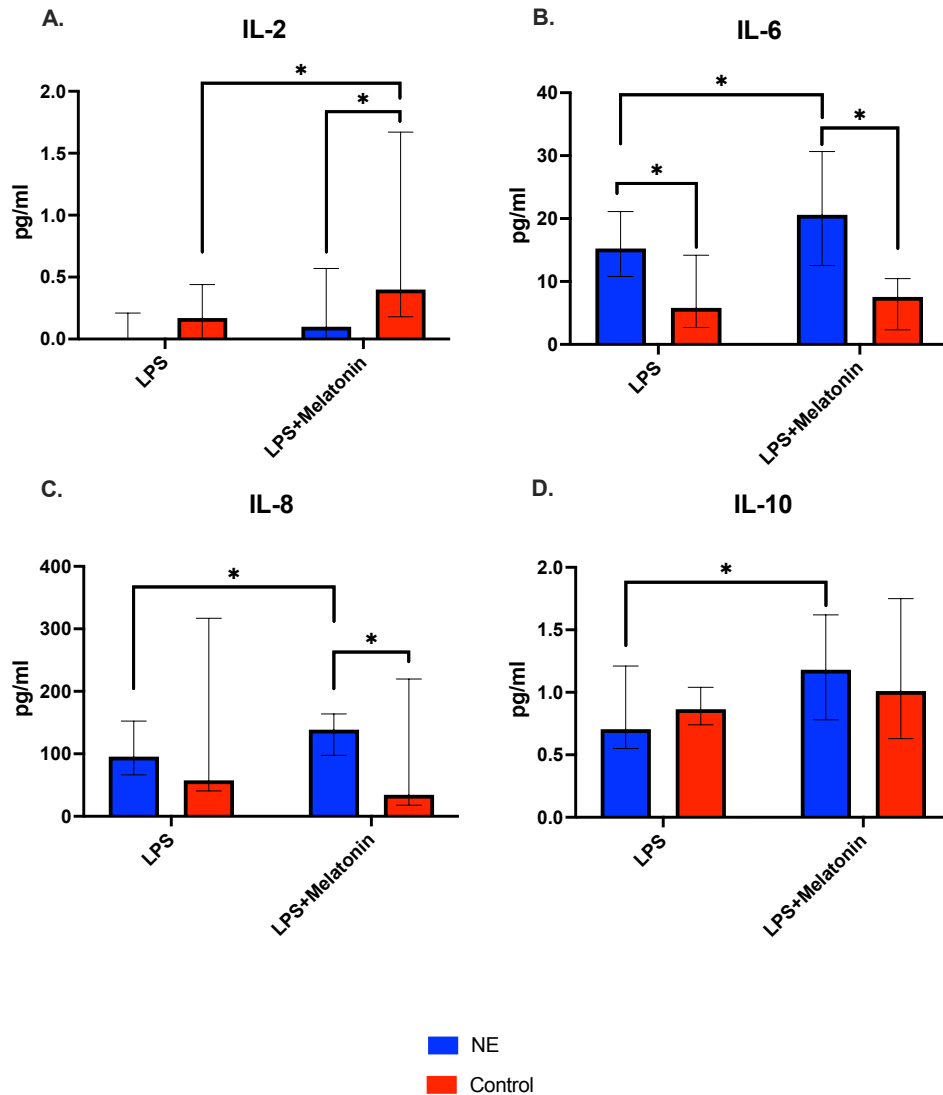
IL-2 increased significantly in endotoxin treated samples following melatonin treatment in controls ( $p < 0.001$ ) but not in infants with NE ( $p = 0.064$ ) (Figure 70A). IL-2 was significantly lower in infants with NE in endotoxin & melatonin treated samples ( $p = 0.010$ ) but not in endotoxin treated samples ( $p = 0.563$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.284$ ) on endotoxin treated IL-2 concentration.

IL-6 increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.005$ ) but not in controls ( $p = 0.687$ ) (Figure 70B). IL-6 was significantly higher in infants in NE in both endotoxin treated ( $p = 0.011$ ) and endotoxin & melatonin treated samples ( $p < 0.001$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.536$ ) on endotoxin treated IL-6 concentration.

IL-8 increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.048$ ) but not in controls ( $p = 0.149$ ) (Figure 70C). IL-8 was significantly higher in infants with NE in endotoxin & melatonin treated samples ( $p = 0.027$ ) but not in endotoxin treated samples ( $p = 0.647$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.004$ ) on endotoxin treated IL-8 concentration.

IL-10 increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.004$ ) but not in controls ( $p = 0.256$ ) (Figure 70D). There was no significant difference in IL-10 concentrations between infants with NE or controls in

endotoxin treated samples ( $p = 0.563$ ) or endotoxin & melatonin treated samples ( $p = 0.967$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.819$ ) on endotoxin treated IL-10 concentration.



**Figure 70** The effect of endotoxin-stimulation (LPS) and melatonin treatment on type 1 (haematopoietin) receptor family cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased IL-2 in controls but not in infants with NE (A). Melatonin treatment following endotoxin-stimulation resulted in increased IL-6, IL-8, and IL-10 in infants with NE but not in controls (B -D). IL-2 was higher in controls following endotoxin & melatonin treatment but there was no difference between groups in endotoxin-stimulated samples (A). IL-6 was higher in infants with NE in both endotoxin-stimulated and endotoxin & melatonin treated samples (B). IL-8 was higher in infants with NE following endotoxin & melatonin treatment but there was no difference between groups in endotoxin-stimulated samples (C). There was no difference between infants with NE and controls in either endotoxin-stimulated samples or endotoxin & melatonin treated samples (D). IL – interleukin.

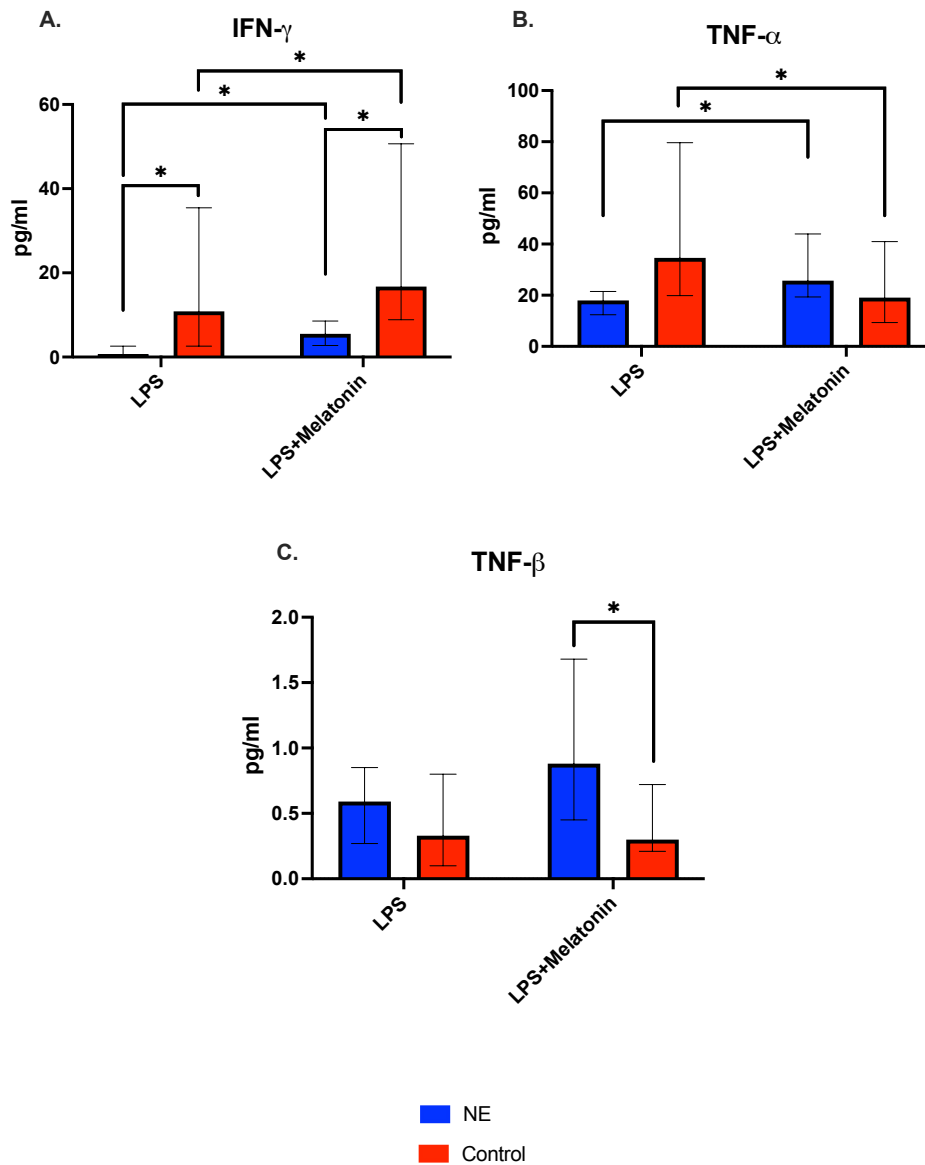
\* indicates a significant difference between treatment or group, with a  $p$  value of  $<0.05$

Type 2 (interferon) receptor family & tumour necrosis factor family cytokines

IFN- $\gamma$  increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p < 0.001$ ) and in controls ( $p = 0.044$ ) (Figure 71A). IFN- $\gamma$  was significantly higher in infants in NE in both endotoxin treated ( $p < 0.001$ ) and endotoxin & melatonin treated samples ( $p = 0.001$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.048$ ) on endotoxin treated IFN- $\gamma$  concentration.

TNF- $\alpha$  increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.005$ ) and decreased significantly in controls ( $p = 0.049$ ) (Figure 71B). There was no significant difference in TNF- $\alpha$  concentrations between infants with NE or controls in endotoxin treated samples ( $p = 0.061$ ) or endotoxin & melatonin treated samples ( $p = 0.132$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p < 0.001$ ) on endotoxin treated TNF- $\alpha$  concentration.

No significant difference was found in endotoxin treated TNF- $\beta$  following melatonin treatment in infants with NE or controls (Figure 71C). TNF- $\beta$  was significantly higher in infants with NE in endotoxin & melatonin treated samples ( $p = 0.005$ ) but not in endotoxin treated samples ( $p = 0.201$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.121$ ) on endotoxin treated TNF- $\beta$  concentration.



**Figure 71** The effect of endotoxin-stimulation (LPS) and melatonin treatment on endotoxin-stimulated type 1 (haematopoietin) receptor family cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased IFN- $\gamma$  in infants with NE and in controls (A). Melatonin treatment following endotoxin-stimulation resulted in increased TNF- $\alpha$  in infants with NE and decreased TNF- $\alpha$  in controls (B). IFN- $\gamma$  was higher in controls in both endotoxin-stimulated and endotoxin & melatonin treated samples (A). TNF- $\beta$  was higher in infants with NE in endotoxin & melatonin treated samples but not in endotoxin-stimulated samples (C). IFN – interferon; TNF – tumour necrosis factor.

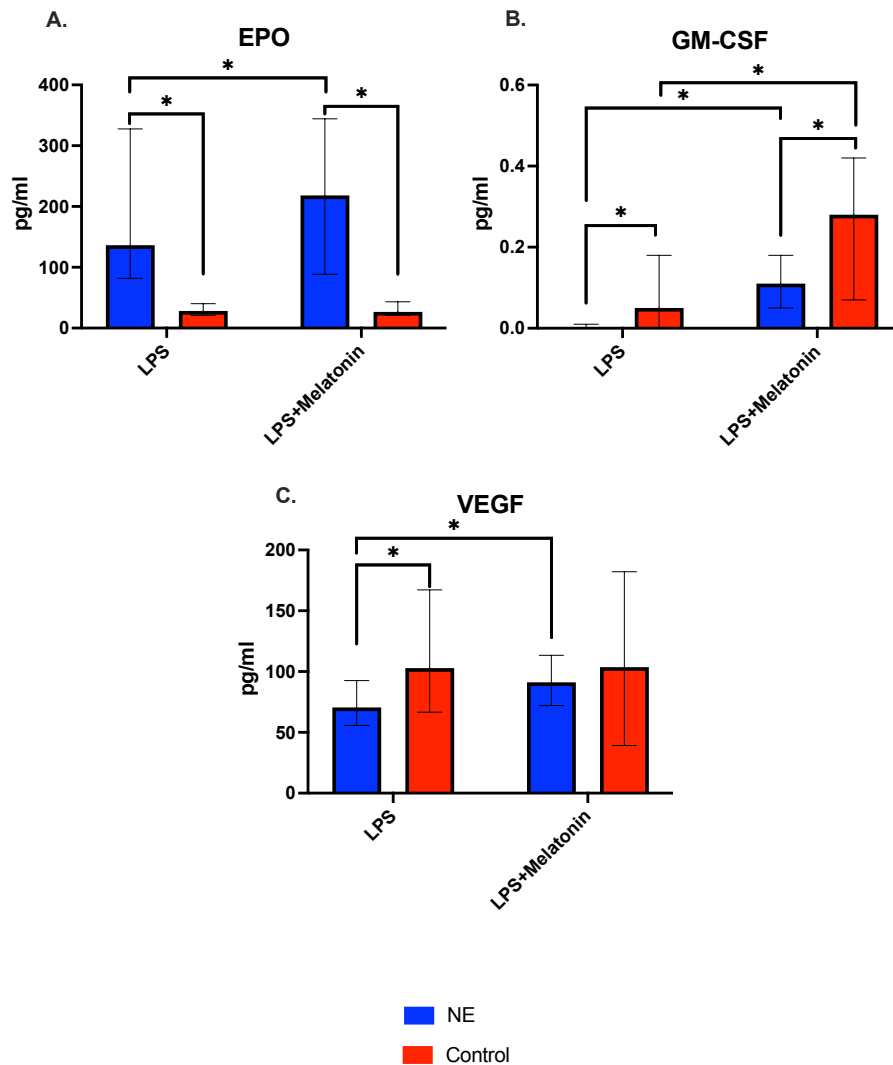
\* indicates a significant difference between treatment or group, with a p value of <0.05

Haematopoiesis and angiogenesis stimulating cytokines

EPO increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.013$ ) but not in controls ( $p = 0.381$ ) (Figure 72A). Epo was significantly higher in infants in NE in both endotoxin treated ( $p < 0.001$ ) and endotoxin & melatonin treated samples ( $p < 0.001$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.225$ ) on endotoxin treated Epo concentration.

GM-CSF increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p < 0.001$ ) and in controls ( $p < 0.001$ ) (Figure 72B). GM-CSF was significantly lower in infants in NE in both endotoxin treated ( $p = 0.047$ ) and endotoxin & melatonin treated samples ( $p = 0.043$ ). There was no significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.953$ ) on endotoxin treated GM-CSF concentration.

VEGF increased significantly in endotoxin treated samples following melatonin treatment in infants with NE ( $p = 0.003$ ) but not in controls ( $p = 0.687$ ) (Figure 72C). VEGF was significantly lower in infants with NE in endotoxin treated samples ( $p = 0.033$ ) but not in endotoxin & melatonin treated samples ( $p = 0.645$ ). There was a significant interaction between the presence of NE and response to melatonin treatment ( $p = 0.043$ ) on endotoxin treated VEGF concentration.



**Figure 72** The effect of endotoxin-stimulation (LPS) and melatonin treatment on haematopoiesis and angiogenesis stimulating cytokines in infants with NE and controls. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased EPO and VEGF in infants with NE but not in controls (A & C). Melatonin treatment following endotoxin-stimulation resulted in increased GM-CSF in both infants with NE and in controls (B). EPO was higher in controls both following endotoxin-stimulation and following endotoxin & melatonin treatment (A). GM-CSF was higher in controls following endotoxin-stimulation and following endotoxin & melatonin treatment (B). VEGF was higher in controls following endotoxin-stimulation but not following endotoxin and melatonin treatment (C). EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; VEGF – vascular endothelial growth factor.

\* indicates a significant difference between treatment or group, with a  $p$  value of  $<0.05$



## Diurnal variation in cytokines concentrations and melatonin treatment response in infants with NE

Circadian variation in cytokines was examined by comparing group differences in cytokine levels between those measured from 10am to 10pm (day) and from 10pm to 10am (night). This time difference was chosen as previous studies have demonstrated distinct diurnal rhythms, with peak cytokine concentration generally occurring at night or in the early morning [453, 460], although there may be variation between cytokines [461]. Results of the distribution of cytokine concentration in day and night for both vehicle and melatonin treated samples from infants with NE were analysed in the same manner as total vehicle and melatonin sample results, by visual inspection of histogram graphs of the distribution of results and confirmation by Shapiro-Wilk tests. The results were not normally distributed, however all were included in the final analysis as any outliers could not be explained by clinical or methodological variability. Repeat samples for some infants were obtained and all results were included in this analysis as it was not possible to match repeat patient samples by time of day of sampling, and including all samples gives the best representation of the data obtained. Comparison with control results was not possible due to insufficient night sampling in control patients.

### *IL-1 receptor family cytokines*

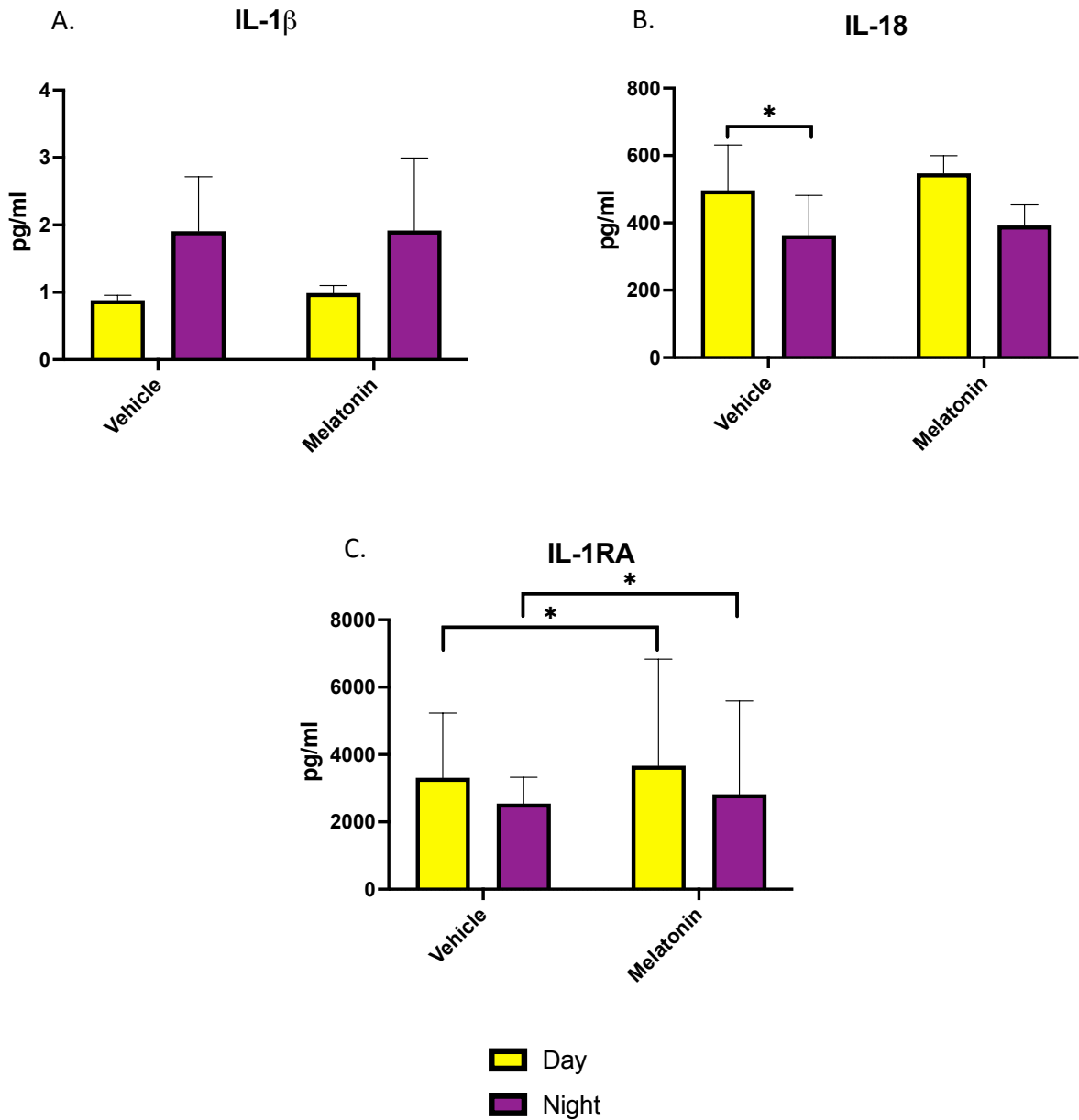
No significant difference was found in IL-1 $\beta$  in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 73A).

There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.861$ ) on IL-1 $\beta$  concentration.

IL-18 was significantly higher in vehicle samples taken during the day than at night ( $p = 0.032$ ) but there was no significant difference in melatonin treated samples (Figure 73B).

There was no significant difference following melatonin treatment in either samples taken during the day or samples taken at night. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.596$ ) on IL-18 concentration.

IL-1RA increased significantly following melatonin treatment in both samples taken during the day ( $p < 0.001$ ) and samples taken at night ( $p = 0.022$ ) (Figure 73C). There were no significant differences in IL-1RA concentrations in vehicle or melatonin samples taken at night or during the day. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.313$ ) on IL-1RA concentration.



**Figure 73** The effect of diurnal variation and melatonin treatment on IL-1 receptor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in increased IL-1RA in infants with NE in both day and night samples (C). IL-18 was higher in day samples in infants with NE at baseline, but not following melatonin treatment (B). IL – interleukin.

\* indicates a significant difference between treatment or group, with a p value of <0.05

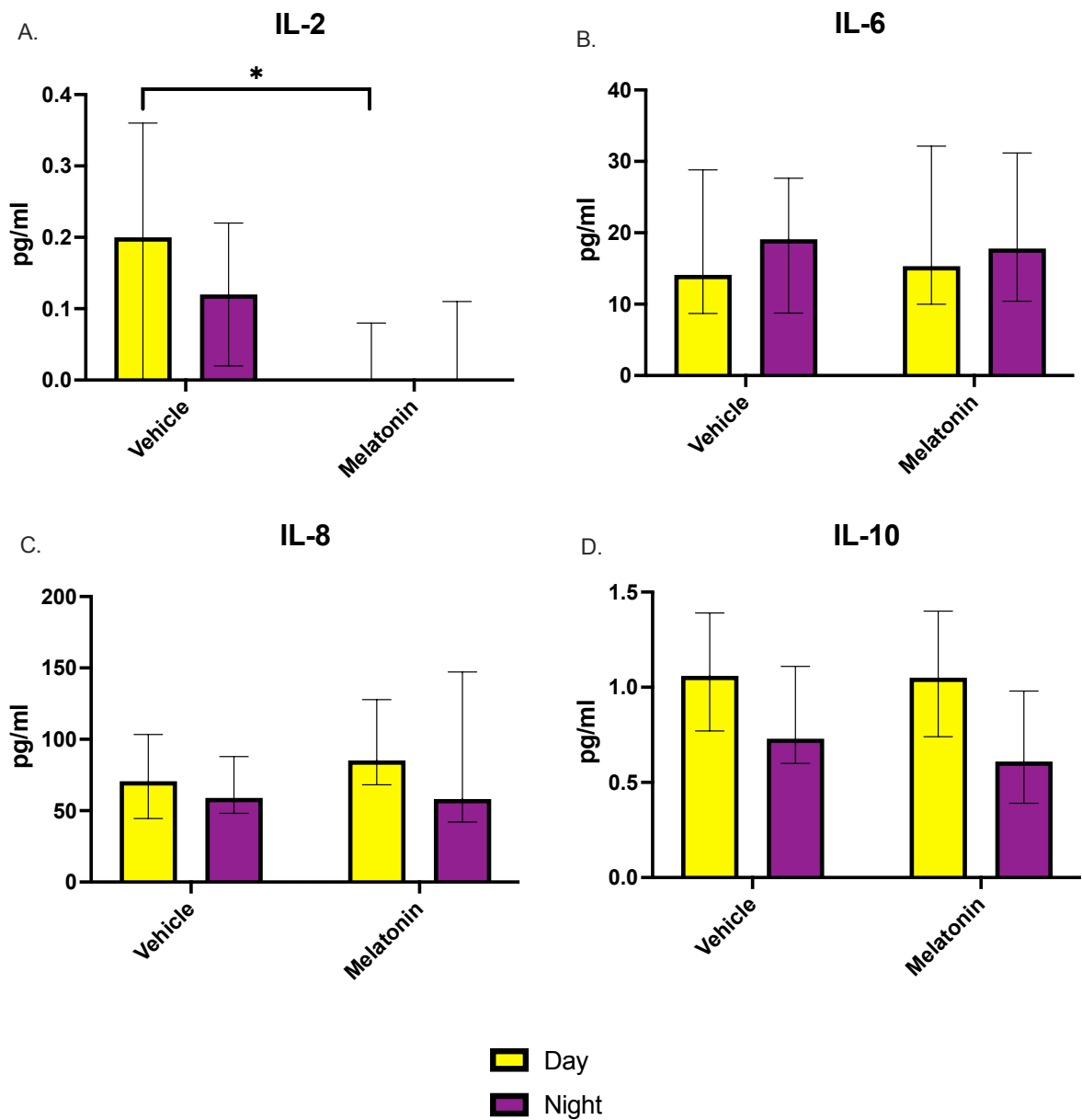
Type 1 (haematopoietin) receptor family cytokines

IL-2 decreased significantly following melatonin treatment in infants with NE in samples taken during the day ( $p = 0.016$ ) but not at night (Figure 74A). No significant difference was found in IL-2 in vehicle or melatonin treated samples taken during the day or at night. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.253$ ) on IL-18 concentration.

No significant difference was found in IL-6 in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 74B). There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.740$ ) on IL-6 concentration.

No significant difference was found in IL-8 in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 74C). There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.993$ ) on IL-8 concentration.

No significant difference was found in IL-10 in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 74D). There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.961$ ) on IL-10 concentration.



**Figure 74** The effect of diurnal variation and melatonin treatment on type 1 (haematopoietin) receptor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in increased IL-2 in infants with NE in day but not in night samples (A). IL – interleukin.

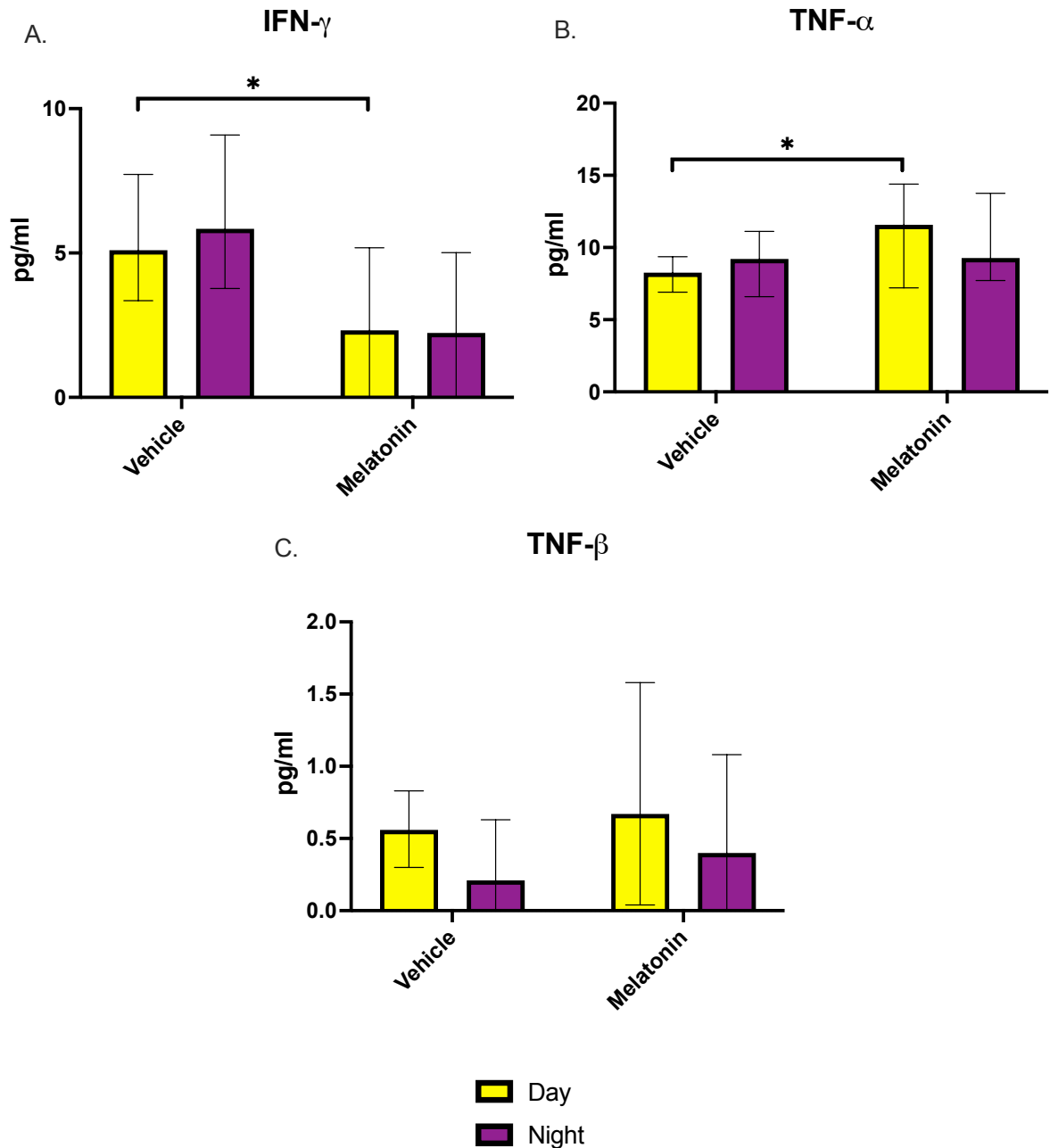
\* indicates a significant difference between treatment or group, with a *p* value of <0.05

Type 2 (interferon) receptor family & tumour necrosis factor family cytokines

IFN- $\gamma$  decreased significantly following melatonin treatment in infants with NE in samples taken during the day ( $p < 0.001$ ) but not at night (Figure 75A). No significant difference was found in IFN- $\gamma$  in vehicle or melatonin treated samples taken during the day or at night. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.291$ ) on IFN- $\gamma$  concentration.

TNF- $\alpha$  increased significantly following melatonin treatment in infants with NE in samples taken during the day ( $p = 0.020$ ) but not at night (Figure 75B). No significant difference was found in TNF- $\alpha$  in vehicle or melatonin treated samples taken during the day or at night. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.691$ ) on TNF- $\alpha$  concentration.

No significant difference was found in TNF- $\beta$  in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 75C). There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.891$ ) on TNF- $\beta$  concentration.



**Figure 75** The effect of diurnal variation and melatonin treatment on type 2 (interferon) receptor family & tumour necrosis factor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in decreased IFN- $\gamma$  in infants with NE in day but not in night samples (A) and increased TNF- $\alpha$  in day but not in night samples (B). IFN – interferon; TNF – tumour necrosis factor.

\* indicates a significant difference between treatment or group, with a *p* value of <0.05

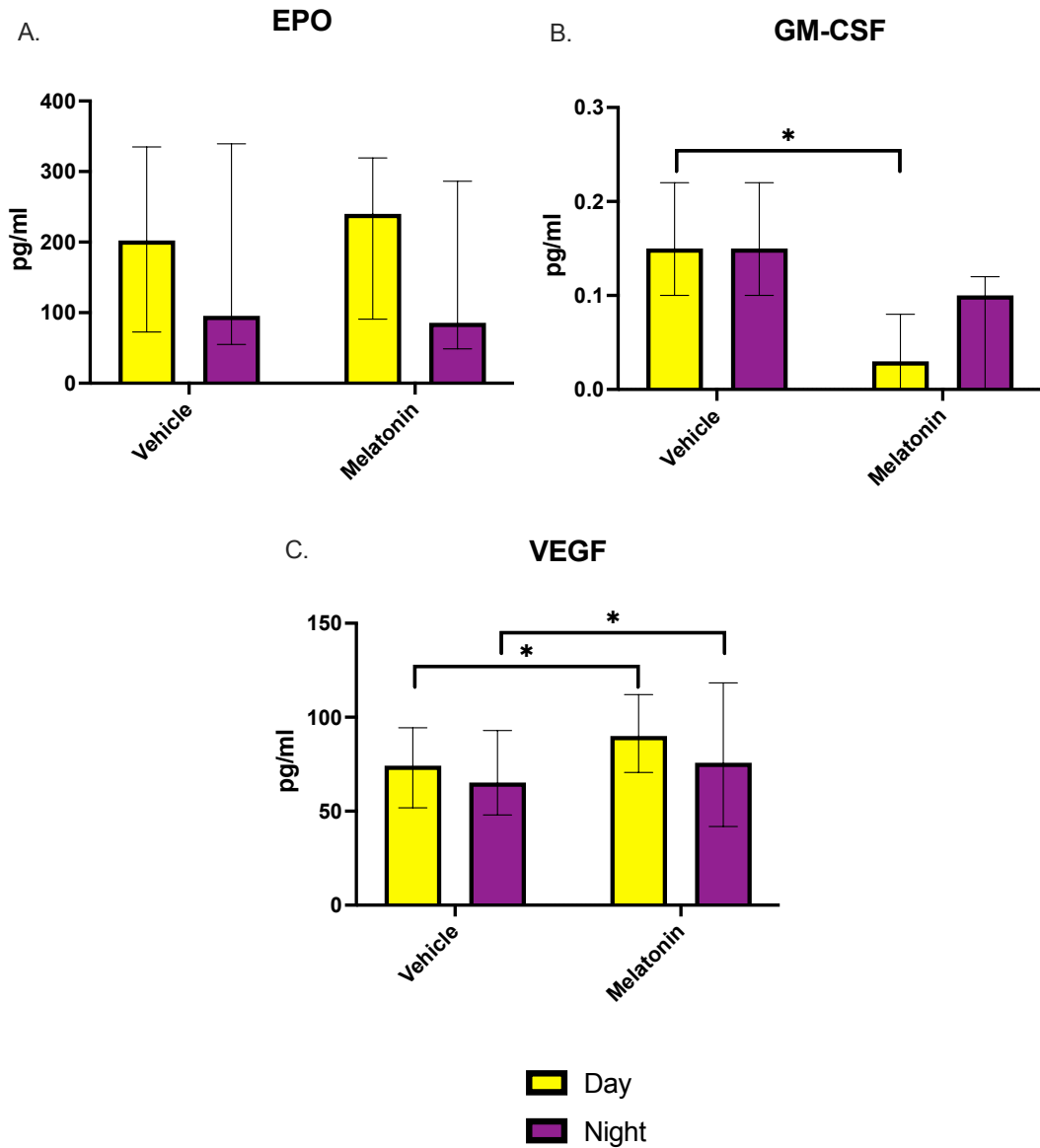
### Haematopoiesis and angiogenesis stimulating cytokines

No significant difference was found in EPO in vehicle or melatonin treated samples taken during the day or at night, nor was any significant difference found in melatonin treated samples compared to vehicle samples taken during the day or at night (Figure 76A). There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.721$ ) on EPO concentration.

GM-CSF decreased significantly following melatonin treatment in infants with NE in samples taken during the day ( $p < 0.001$ ) but not at night (Figure 76B). No significant difference was found in GM-CSF in vehicle or melatonin treated samples taken during the day or at night. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.171$ ) on GM-CSF concentration.

VEGF increased significantly following melatonin treatment in both samples taken during the day ( $p = 0.003$ ) and samples taken at night ( $p = 0.035$ ) (Figure 76C). There were no significant differences in VEGF concentrations in vehicle or melatonin samples taken at night or during the day. There was no significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.703$ ) on VEGF concentration.





**Figure 76** The effect of diurnal variation and melatonin treatment on haematopoiesis and angiogenesis stimulating cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment resulted in decreased GM-CSF in infants with NE in day but not in night samples (B) and increased VEGF in both day and night samples (C). IFN – interferon; TNF – tumour necrosis factor. EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; VEGF – vascular endothelial growth factor.

\* indicates a significant difference between treatment or group, with a *p* value of <0.05

## Diurnal variation in cytokines and melatonin treatment response in endotoxin-stimulated samples from infants with NE

Results of the distribution of cytokine concentration in endotoxin treated and endotoxin & melatonin treated samples from infants with NE were analysed in the same manner as day and night vehicle and melatonin sample results, by visual inspection of histogram graphs of the distribution of results and confirmation by Shapiro-Wilk tests. The results were not normally distributed, however all were included in the final analysis as any outliers could not be explained by clinical or methodological variability. Repeat samples for some infants were obtained and all results were included in this analysis as it was not possible to match repeat patient samples by time or day of sampling and including all samples gives the best representation of the data obtained.

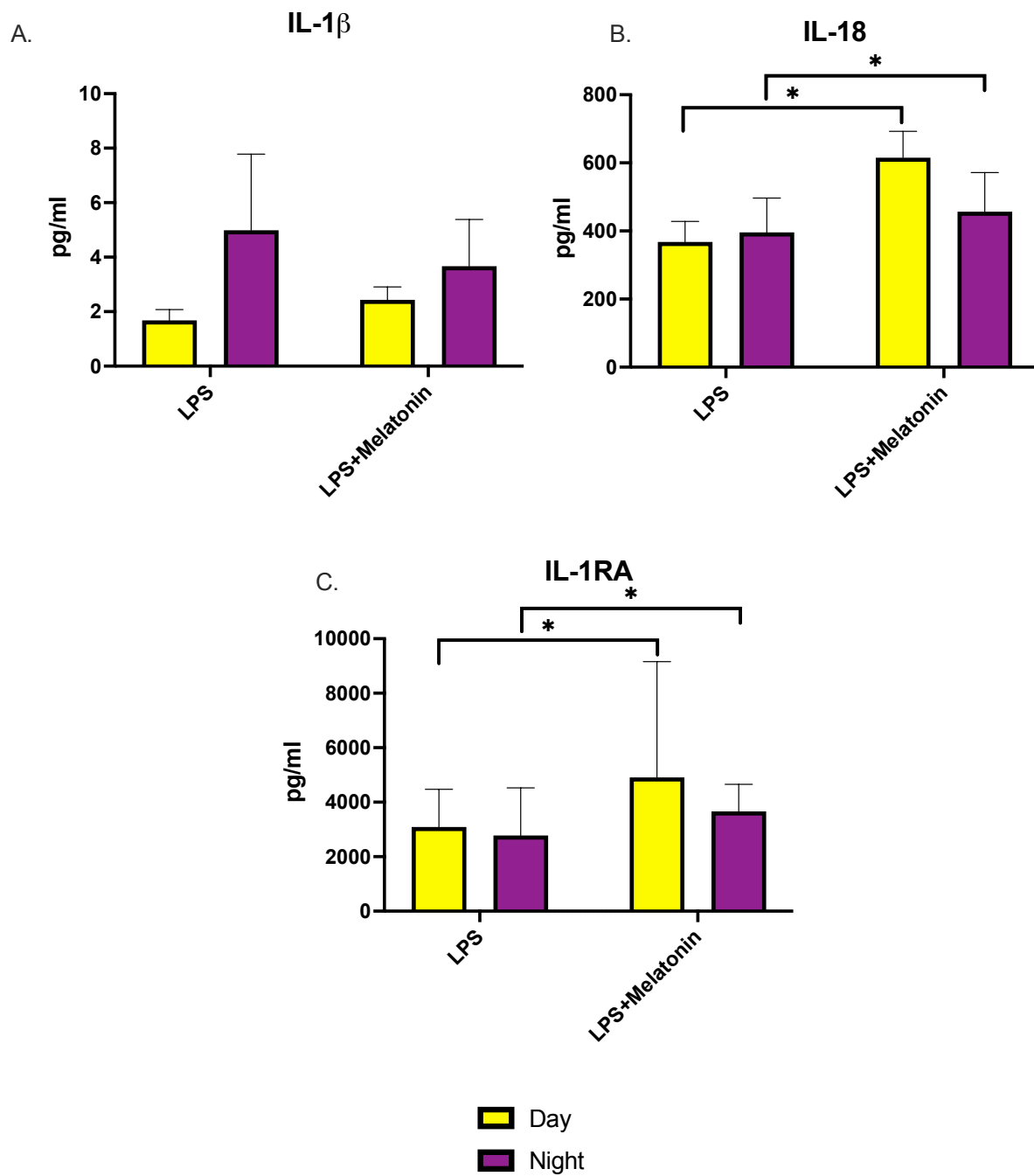
### *IL-1 receptor family cytokines*

No significant difference was found in IL-1 $\beta$  in endotoxin treated or endotoxin and melatonin treated samples taken during the day or at night, nor was any significant difference found in endotoxin and melatonin treated samples compared to endotoxin treated samples taken during the day or at night (Figure 77A). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.816$ ) on IL-1 $\beta$  concentration.

There were no significant differences in IL-18 concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. IL-18 increased significantly following melatonin treatment in endotoxin treated samples in both samples

taken during the day ( $p < 0.001$ ) and samples taken at night ( $p = 0.004$ ) (Figure 77B). There was a significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.002$ ) on IL-18 concentration.

There were no significant differences in IL-1RA concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. IL-1RA increased significantly following melatonin treatment in endotoxin treated samples in both samples taken during the day ( $p < 0.001$ ) and samples taken at night ( $p = 0.015$ ) (C). There was a significant interaction between the timing of sampling and response to melatonin treatment (Figure 77C) following endotoxin stimulation ( $p = 0.003$ ) on IL-1RA concentration.



**Figure 77** The effect of diurnal variation and endotoxin-stimulated or endotoxin & melatonin treatment on IL-1 receptor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased IL-18 and IL-1RA in infants with NE in both day and night samples (B & C). IL – interleukin.

\* indicates a significant difference between treatment or group, with a *p* value of <0.05

Type 1 (haematopoietin) receptor family cytokines

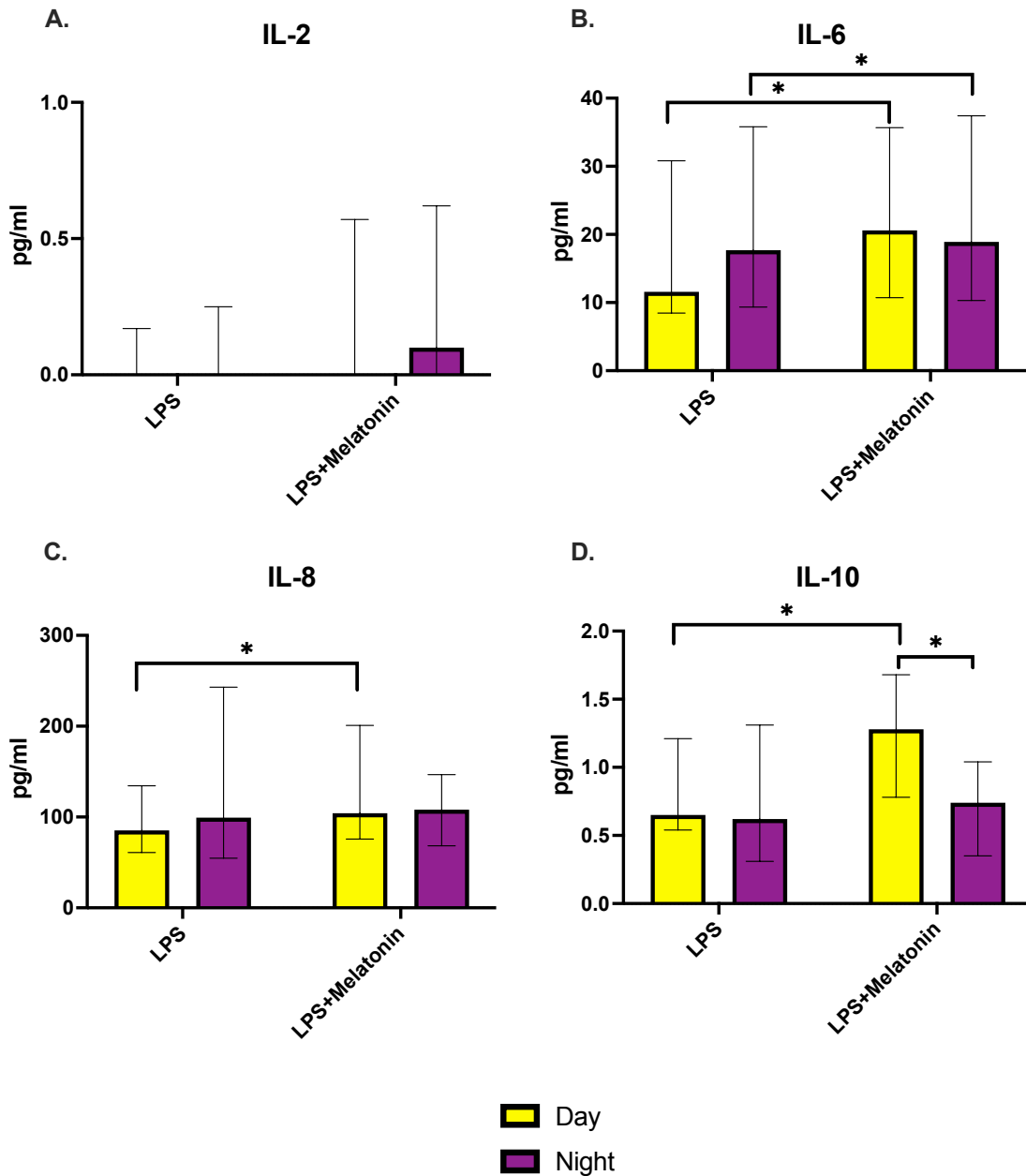
No significant difference was found in IL-2 in endotoxin treated or endotoxin & melatonin treated samples taken during the day or at night, nor was any significant difference found in endotoxin & melatonin treated samples compared to endotoxin treated samples taken during the day or at night (Figure 78A). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.774$ ) on IL-2 concentration.

There were no significant differences in IL-6 concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. IL-6 increased significantly following melatonin treatment in endotoxin treated samples in both samples taken during the day ( $p < 0.001$ ) and samples taken at night ( $p = 0.011$ ) (Figure 78B). There was a significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.002$ ) on IL-6 concentration.

There were no significant differences in IL-8 concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. IL-8 increased significantly following melatonin treatment in endotoxin treated samples in samples taken during the day ( $p = 0.007$ ) but not in samples taken at night (Figure 78C). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.146$ ) on IL-8 concentration.

IL-10 was significantly higher in endotoxin & melatonin treated samples taken during the day than at night ( $p = 0.033$ ) but there was no significant difference in endotoxin treated samples. IL-10 increased significantly following melatonin treatment in endotoxin

treated samples in samples taken during the day ( $p = <0.001$ ) but not in samples taken at night (Figure 78D). There was a significant interaction between the timing of sampling and response to melatonin treatment ( $p = 0.020$ ) on IL-10 concentration.



**Figure 78** The effect of diurnal variation and endotoxin-stimulated or endotoxin & melatonin treatment on type 1 (haematopoietin) receptor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased IL-6 in infants with NE in both day and night samples (B). Melatonin treatment resulted in increased IL-8 and IL-10 in infants with NE in day but not in night samples (C & D). IL-10 was higher in infants with NE in day samples following endotoxin & melatonin treatment but not in endotoxin-stimulated samples (D). IL – interleukin.

\* indicates a significant difference between treatment or group, with a  $p$  value of  $<0.05$

Type 2 (interferon) receptor family & tumour necrosis factor family cytokines

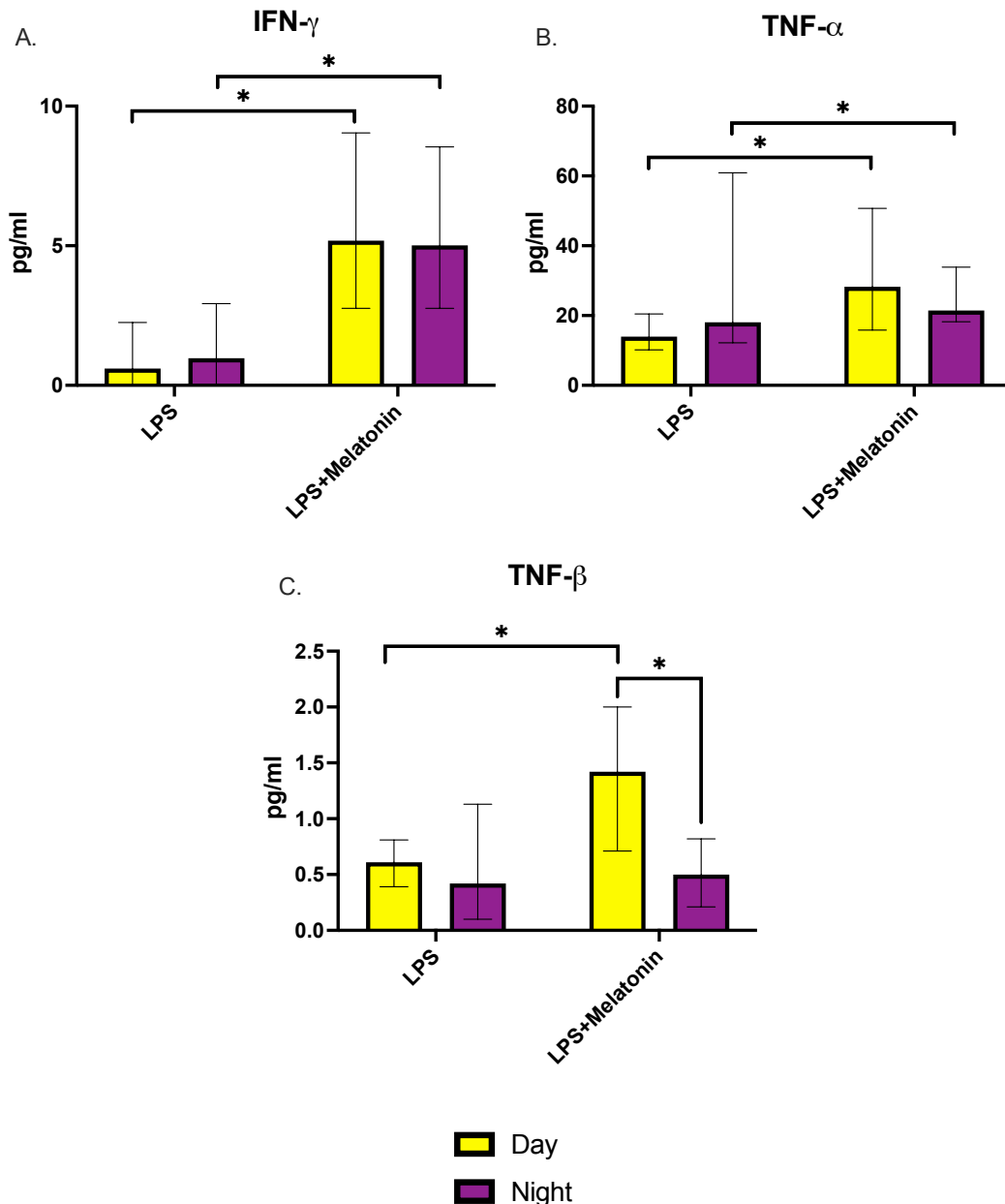
There were no significant differences in IFN- $\gamma$  concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. IFN- $\gamma$  increased significantly following melatonin treatment in endotoxin treated samples in both samples taken during the day ( $p < 0.001$ ) and in samples taken at night ( $p = 0.016$ ) (Figure 79A).

There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.225$ ) on IFN- $\gamma$  concentration.

There were no significant differences in TNF- $\alpha$  concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. TNF- $\alpha$  increased significantly following melatonin treatment in endotoxin treated samples in both samples taken during the day ( $p < 0.001$ ) and in samples taken at night ( $p = 0.044$ ) (Figure 79B). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.405$ ) on TNF- $\alpha$  concentration.

TNF- $\beta$  concentrations were significantly higher in endotoxin-stimulated and melatonin treated samples taken at night ( $p = 0.013$ ), but not in samples taken during the day (Figure 79C). TNF- $\beta$  increased significantly following melatonin treatment in endotoxin treated samples in samples taken during the day ( $p = 0.009$ ) but not in samples taken at night. There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.405$ ) on TNF- $\beta$  concentration.





**Figure 79** The effect of diurnal variation and endotoxin-stimulated or endotoxin & melatonin treatment on type 2 (interferon) receptor family & tumour necrosis factor family cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased IFN- $\gamma$  and TNF- $\alpha$  in infants with NE in both day and night samples (A & B). Melatonin treatment resulted in increased TNF- $\beta$  in infants with NE in day but not in night samples (C). TNF- $\beta$  was higher in infants with NE in day samples following endotoxin & melatonin treatment but not in endotoxin-stimulated samples (C). IFN – interferon; TNF – tumour necrosis factor.

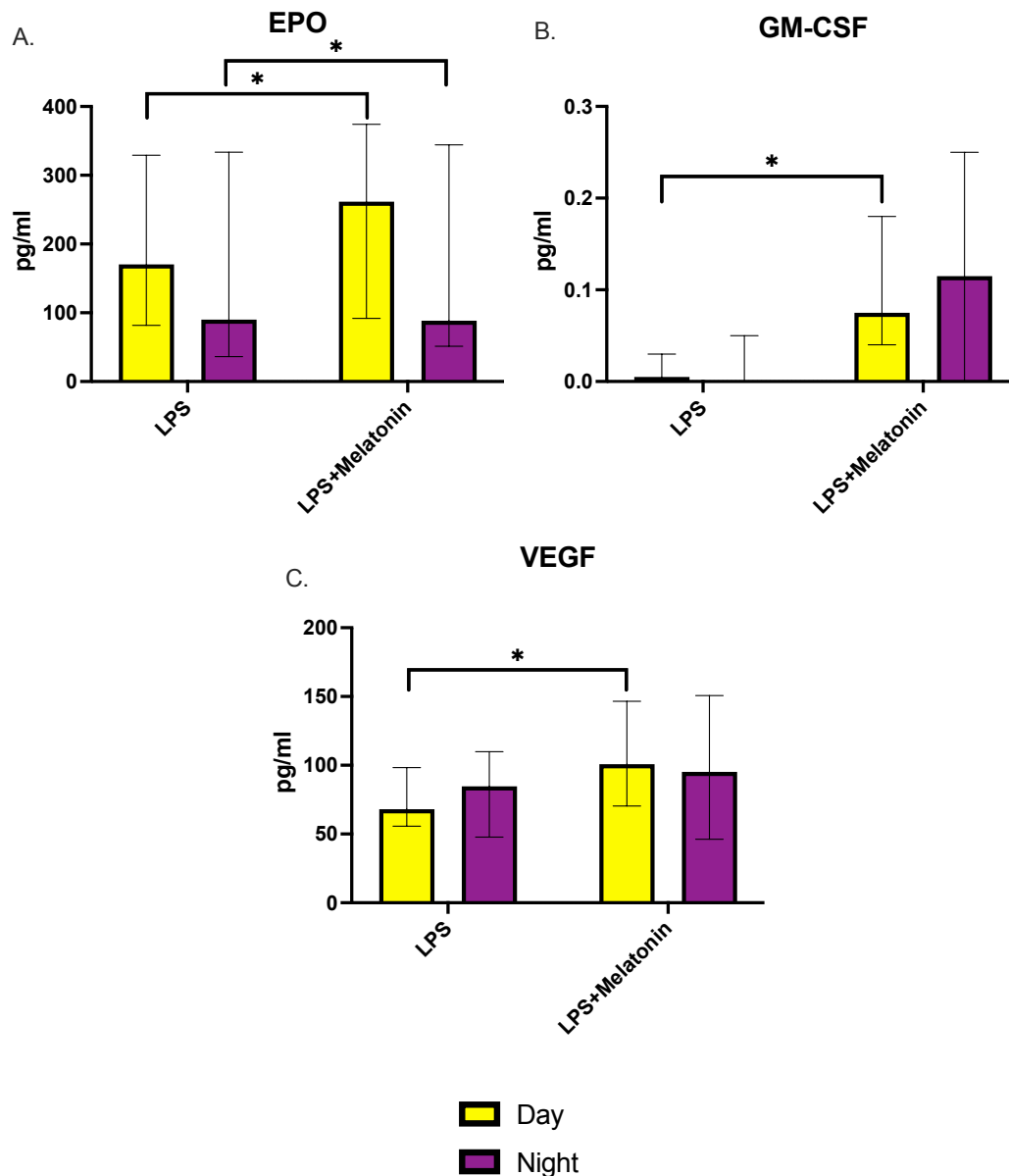
\* indicates a significant difference between treatment or group, with a *p* value of <0.05

### Haematopoiesis and angiogenesis stimulating cytokines

There were no significant differences in EPO concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. EPO increased significantly following melatonin treatment in endotoxin treated samples in both samples taken during the day ( $p < 0.001$ ) and samples taken at night ( $p = 0.034$ ) (Figure 80A). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.114$ ) on EPO concentration.

There were no significant differences in GM-CSF concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. GM-CSF increased significantly following melatonin treatment in endotoxin treated samples in samples taken during the day ( $p < 0.001$ ) but not in samples taken at night (Figure 80B). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.15$ ) on GM-CSF concentration.

There were no significant differences in VEGF concentrations in endotoxin treated or endotoxin & melatonin treated samples taken at night or during the day. VEGF increased significantly following melatonin treatment in endotoxin treated samples in samples taken during the day ( $p < 0.001$ ) but not in samples taken at night (Figure 80C). There was no significant interaction between the timing of sampling and response to melatonin treatment following endotoxin stimulation ( $p = 0.1$ ) on VEGF concentration.



**Figure 80** The effect of diurnal variation and endotoxin-stimulated or endotoxin & melatonin treatment on haematopoiesis and angiogenesis stimulating cytokines in infants with NE. Results (median and interquartile range) are measured in pg/ml. Melatonin treatment following endotoxin-stimulation resulted in increased EPO in infants with NE in both day and night samples (A). Melatonin treatment resulted in increased GM-CSF and VEGF in infants with NE in day but not in night samples (B & C). EPO – erythropoietin; GM-CSF – granulocyte-macrophage colony-stimulating factor; VEGF – vascular endothelial growth factor.

\* indicates a significant difference between treatment or group, with a *p* value of <0.05

## Discussion

We found several differences in cytokines at baseline for infants with NE compared to controls. Pro-inflammatory cytokines IL-6, IL-8, IL-18, TNF- $\alpha$ , TNF- $\beta$  and anti-inflammatory cytokine Epo were all increased in infants with NE compared to controls, and the pro-inflammatory cytokine IFN- $\gamma$  was lower in infants with NE compared to controls. A recent study of differences in cytokines in the first 4 days of life in NE compared to healthy controls demonstrated similar results, with increased IL-6, IL-1RA, and Epo in NE compared to controls, but lower VEGF [450]. Much focus on immune modulation in NE has been on changes in IL-1 $\beta$  as it has such a profound inflammatory effect [462]. However, neither our study nor the study by O’Dea, nor our meta-analysis of IL-1 $\beta$  (see Chapter 4), identified any significant differences between controls and infants with NE in levels of this cytokine, nor as a prognostic factor for NE. This finding is not in keeping with pre-clinical models of NE, which have identified IL-1 $\beta$  as a key mediator of the inflammatory response in NE and correlated with outcome [463]. Differences that could explain this finding may be that IL-1 $\beta$  is increased in CSF but not in serum, or that there appears to be an important temporal relationship between NE and the rise in IL-1 $\beta$  which may not be captured from these studies which collect samples over different time points, or that TH modulates the release of IL-1 $\beta$  [464]. Early samples collected within the first 24 hours of life have shown elevated IL-1 $\beta$  in infants with NE compared to controls [118] and an association between IL-1 $\beta$  and adverse outcome in NE [101]. However, there was no difference in IL-1 $\beta$  in later samples taken at 72 hours of life between infants with NE and controls [240], or between infants with good or adverse outcomes in NE [101]. Samples reported in our research were taken during the first

week of life and there were insufficient samples taken within the first 24 hours to draw any conclusions regarding the effect of melatonin on cytokine production. Much less is understood about the role of IL-18 in NE compared to IL-1 $\beta$ . Further, differences in the inflammatory signals that regulate the expression of IL-1 $\beta$  and IL-18 are poorly understood [465].

Melatonin treatment resulted in many significant changes in cytokine levels in both NE and controls. Compared to vehicle samples in infants with NE, melatonin treatment resulted in increased anti-inflammatory IL-1RA, and reduced pro-inflammatory IL-2, IFN- $\gamma$ , and GM-CSF. However, melatonin treatment also resulted in increased pro-inflammatory TNF-, IL-8, and VEGF. In controls, melatonin treatment resulted in increased pro-inflammatory IL-18, IL-6, IL-8, IFN- $\gamma$ , and VEGF compared to vehicle samples, and increased anti-inflammatory IL-1RA and Epo. There was a significant interaction between the presence of NE and the response to melatonin treatment in inflammatory cytokines IL-6, IL-10, IL-18, IFN- $\gamma$ , and Epo. Differences in response to melatonin treatment were anticipated, as infants with NE have a dysregulated immune response and are undergoing an immune-modulating therapy in TH [107]. Melatonin treatment did not result in a significant difference in IL-1 $\beta$  in either infants with NE or controls. Nor did melatonin treatment result in any significant difference in IL-18 in infants with NE, although it did result in an increase in IL-18 in the control group. This is a surprising result as many previous studies have demonstrated that melatonin treatment leads to inhibition of NLRP3 inflammasome activation and thus reduced secretion of IL-1 $\beta$  [466]. This is one of the hypothesised beneficial anti-

inflammatory properties of melatonin in NE [181]. However, the immune-modulating effects of melatonin are conditional on the clinical context, and it is also known to have immune-stimulating properties including increased formation of ROS and increased release of pro-inflammatory cytokines including IL-1 $\beta$  in monocytes, macrophages and T helper cells [108]. The effect of melatonin on cytokine release is dependent on specific melatonin receptor activation and this remains unexplored in the neonatal population and an area of ongoing research [108]. Similarly, melatonin modulates TNF- $\alpha$  production through NF $\kappa$ B regulation [467], and reduces NF $\kappa$ B and TNF- $\alpha$  in clinical trials of NE [118, 468]. Melatonin treatment increased IL-6 in controls, but not in infants with NE, although IL-6 is higher in infants with NE compared to controls at baseline. Elevated IL-6 is associated with increased risk of adverse outcome in NE [101]. The effects of melatonin treatment on IL-6 levels, are again context-specific, and will be influenced by the presence of sepsis or sterile inflammation [469, 470]. Melatonin treatment resulted in a reduction in IL-2 in NE but there was no significant difference in controls. This is again an unexpected finding as melatonin has previously been shown to enhance IL-2 production [471]. However, most studies of the effect of melatonin on IL-2 production to date have focused on the role of melatonin in cancer immunotherapy as an adjunctive treatment to IL-2 therapy, and in this context melatonin is known to exhibit immune-stimulating effects for anti-cancer cells [472]. Further, the effects of melatonin on IL-2 production are melatonin receptor dependent and not fully understood at present [473]. The relationship between IL-2 and melatonin is regulated by IL-12 which was not measured in our research [474].

In endotoxin-stimulated samples patients with NE had increased levels of IL-1RA, IL-6, IL-18, and Epo, and decreased levels of GM-CSF, IFN- $\gamma$ , and VEGF. This demonstrates similarities to a recent publication which demonstrated decreased GM-CSF, IFN- $\gamma$ , and VEGF in infants with NE compared to controls, but also decreased IL-2, IL-6, IL-8, and TNF- $\alpha$  [450]. This study demonstrated a significant temporal relationship with response to endotoxin stimulation which may explain some of the differences in cytokine response in this study. Melatonin treatment following endotoxin stimulation resulted in increased pro-inflammatory GM-CSF, IL-6, IL-8, IL-18, IFN- $\gamma$ , TNF- $\alpha$ , and VEGF, and increased anti-inflammatory IL-1RA, IL-10, and Epo in infants with NE. In controls, melatonin treatment following endotoxin stimulation resulted in increased pro-inflammatory GM-CSF, IL-2, IFN- $\gamma$ , and TNF- $\alpha$ . We found a significant interaction between the presence of NE and the response to melatonin treatment following endotoxin-stimulation in cytokines IL-8, IL-18, IFN- $\gamma$ , TNF- $\alpha$ , and VEGF. Melatonin treatment resulting in significant immune stimulation in both infants with NE and controls in endotoxin-stimulated samples, however the effect was much more pronounced in patients with NE with a significant increase in many more cytokines compared to controls. It is unsurprising that melatonin treatment would have a different response in NE and controls given that patients with NE have dysregulated inflammatory response, some have a clinical diagnosis of sepsis and there are major differences in the inflammatory response sterile and non-sterile inflammatory response in NE, that infants with NE are undergoing an immune-modulating treatment in TH, and that the inflammatory response to melatonin treatment is context specific. Nonetheless these results are surprising as melatonin has been demonstrated to mitigate LPS-induced

neutrophil dysfunction [454] and inhibits LPS-induced chemokine gene expression [475]. A RCT of melatonin treatment in neonatal sepsis demonstrated reduced inflammatory biomarker response and improved outcomes [436, 476].

We did not find significant diurnal variation in vehicle sample cytokines in NE. IL-18 was lower at night in vehicle samples compared to day samples in patients with NE. In adults, distinct diurnal rhythms in inflammatory cytokine concentration for several inflammatory cytokines including IL-1, TNF- $\alpha$ , and IFN- $\gamma$  has been demonstrated [453], however we could not find any study that examined this question in the neonatal population. Circadian rhythms emerge gradually in the postnatal period [85] and it remains unclear whether there is a distinct diurnal rhythm of serum cytokine concentrations in the neonatal population. This is a crucial question, as improved understanding of the circadian effects of inflammation have been implemented in several diseases of adulthood such as rheumatoid arthritis [477], and chronotherapeutic approaches have been proposed in NE [90].

We found diurnal variation in response to melatonin treatment compared to vehicle samples for several inflammatory cytokines in patients with NE. Anti-inflammatory IL-1RA and pro-inflammatory VEGF were increased following melatonin treatment in both day and night samples following melatonin treatment. Melatonin treatment resulted in significant changes only in day samples in several cytokines including IL-2, IFN- $\gamma$ , and GM-CSF which were decreased following melatonin treatment, and TNF- $\alpha$  which was increased in day samples but not at night. Potential explanations for these variations include diurnal rhythms



in melatonin receptor expression [478], diurnal variation in other inflammatory processes such as cortisol levels [453] and immune cell function and response [479], and the effect of sleep on the inflammatory response [480]. As discussed above however, the circadian effect of these processes is poorly understood in the neonatal population.

In endotoxin-stimulated samples, there was no significant difference between day and night response to endotoxin-stimulation. However, diurnal variation in response to melatonin treatment in endotoxin treatment samples was evident in cytokines IL-8, IL-10, TNF- $\beta$ , GM-CSF, and VEGF in which melatonin cytokine concentrations were increased in day samples but not at night. In cytokines IL-1RA, IL-18, IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and Epo cytokines were increased in both day and night samples. Previous studies have demonstrated diurnal rhythms in endotoxin tolerance in adult samples [453, 481, 482], however this has not been explored in the neonatal population to our knowledge. For the same reasons outlined above, the circadian influence of inflammatory responses in neonatal populations, and in particular for those with NE, remains poorly understood and requires further exploration.

One of the limitations of this study is lack of matching sampling timepoints for each participant in the study. Several different phases of injury have been identified in NE [420], and each is associated with different aspects of immune dysregulation [19]. We attempted to collect samples as early as possible in the course of treatment for each patient, however this was not possible due to time required for consent prior to enrolment and due to constraints of sampling during phlebotomy for clinical indications. For these reasons samples were collected over the first week of life and may represent various phases of

injury and limiting. Further exploration of our results is required to assess the response to melatonin treatment at different phases of injury. There are many different causes of NE identified [2], and the immune response in NE is affected by the cause preceding hypoxia and ischaemia meaning different therapeutic approaches are required. For example, TH may not be as effective for patients with NE and chorioamnionitis compared to the total cohort of patients with NE [483]. To account for these differences in immune response in NE and the effect of any immunomodulating therapy would require a very large sample size which was not feasible in this study. The limited sample size also limits the interpretation of the circadian effect on cytokines, and it was only possible to analyse 2 different phases within the 24-hour cycle. A further limitation of this study was the lack of night sampling for control patients and consequently the inability to analyse any circadian differences in the healthy neonatal population. As samples were taken opportunistically from healthy controls during phlebotomy for non-urgent indications, all samples were taken during daytime hours.

## Conclusion

Melatonin induces a complex immune-modulating effect in infants with NE undergoing TH in compared to both vehicle and endotoxin-stimulated samples. However, there is a mixed pro- and anti-inflammatory effect of melatonin treatment, and it is difficult to establish a specific pattern of response. Infants with NE were hyporesponsive to melatonin treatment compared to healthy controls, who displayed a more consistent pro-inflammatory response to melatonin treatment. We did not find significant levels of diurnal

variation in many cytokines in NE, nor in response to endotoxin-stimulation nor melatonin treatment.

## Chapter 7: The effect of melatonin treatment on innate immune function in infants with neonatal encephalopathy

### Abstract

**Introduction:** Neonatal encephalopathy (NE) remains one of the major causes of mortality and long-term neurodisability in newborns, despite the use of therapeutic hypothermia (TH) in high resource settings. Therefore, there is an urgent need for adjunctive neuroprotective interventions. One of the main pathological processes in NE is dysregulated inflammation. Melatonin is a potent immune-modulating agent, and has been demonstrated to improve inflammatory regulation and outcomes in pre-clinical models of NE.

**Methods:** Infants with moderate-severe NE who were undergoing TH were prospectively recruited (n=36). Whole blood samples were collected during the first week of life and analysed for neutrophil and monocyte function by flow cytometry (CD11b, TLR2, NOX and intracellular cytokines IL-6, IL-10, IL-17a, TNF- $\alpha$ ), inflammasome (NLRP3, ASC, IL-1 $\beta$ ) and circadian rhythm (BMAL1, CLOCK, CRY, REV-ERB- $\alpha$ ) gene expression by qPCR, microRNA (miR-20a, -20b, -93, 155, -582) gene expression by qPCR, and inflammatory cytokines (EPO, GM-CSF, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1RA, IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-18, TNF- $\alpha$ , TNF- $\beta$ , and VEGF) by ELISA. Samples were analysed following stimulation with LPS (endotoxin) or Pam3CSK4 (simulating gram positive infection), and following treatment with melatonin.

**Results:** Fifty-four infants were enrolled including NE (n=36) and Controls (n=18). Melatonin treatment reduced neutrophil TLR-2 expression in endotoxin-stimulated samples and

increased NLRP3 gene expression compared to untreated samples. There were no effects of melatonin treatment on the expression of intracellular cytokines, circadian rhythm gene expression, or microRNA expression.

**Discussion:** Melatonin altered TLR-2 and inflammasome gene expression without altering cytokine gene expression. This may contribute to the immunomodulatory effect of melatonin and neuroprotection in NE in preliminary human and animal models.

## Introduction

Melatonin is an endogenous hormone produced in the pineal gland in response to darkness stimulation received by the suprachiasmatic nucleus in the hypothalamus [78]. Therefore it is primarily secreted at night and demonstrates significant diurnal variation in serum concentration [484]. It is a highly lipophilic hormone and easily crosses the blood brain barrier [485]. Melatonin is a major immune-modulating agent and it has pleiotropic immune effects including anti-inflammatory, anti-oxidant, and antiapoptotic properties [181]. Melatonin's effects on the immune system are context-specific and it can either promote or suppress inflammatory reactions [108].

Neutrophils and monocytes are key cells of the peripheral innate immune system [486]. They promote inflammatory response to cell damage and pathogens through the production of reactive oxygen species, and inflammatory chemokines and cytokines [36] and induce cell death through a number of mechanisms including apoptosis, pyroptosis and NETosis [487]. They are activated through cell surface antigens call pattern recognition receptors (PRRs), which recognise damage associated molecular patterns triggering an inflammatory reaction [488], including CD11b, a marker of neutrophil activation [489], toll-like receptor (TLR)-2, [490], and NADPH oxidase (NOX) [491]. Dysregulation of neutrophils and monocytes have been implicated in worsening inflammation and outcomes in NE [43, 44]. Melatonin regulates both migration and activation of neutrophils and monocytes [492, 493], and restores dysregulated neutrophil function [454].

Inflammasomes are intracellular multi-protein complexes assembled by pattern-recognition receptors in response to pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS) [494]. They play a critical role in host defence and function of the innate immune system by triggering downstream inflammatory pathways, and their formation is essential in the production of pro-inflammatory cytokines IL-1 $\beta$ . However, their dysregulation has been implicated in the pathogenesis of several inflammatory disorders [495]. Altered inflammasome activation has been implicated in several neonatal conditions including neonatal encephalopathy [496], and this persists into early childhood [497]. Melatonin inhibits NLRP3 inflammasome activation through several mechanisms including inhibition of NF $\kappa$ B signalling and through the suppression of reactive oxygen species (ROS) production, a trigger for NLRP3 activation [498].

Circadian rhythm genes (CRGs) are present in nearly every immune cell and are entrained to the external environment by light stimulus received in the suprachiasmatic nucleus in the hypothalamus . CRGs play a key role in several aspects of innate immunity such as immune cell migration and functions such as cytokine production [64]. It has been hypothesised that circadian disruption may contribute to further dysregulated inflammation and worse outcomes in NE [12, 499]. Potential zeitgebers, entrainment factors, are amenable to improved management and have demonstrated improved outcomes in other neonatal populations [93]. Melatonin administration has been demonstrated to repair CRG disruption and normalise innate immunity and mitochondrial homeostasis [81].

microRNAs are small single-stranded non-coding pieces of RNA [500]. They function by binding to and repressing mRNA translation by regulating post-transcriptional gene expression and they are powerful regulators of various cellular activities [501]. They are key regulators of the immune system by modulating signalling of the onset and termination of inflammation, and they can either promote or suppress inflammation [502]. Alteration of the expression of several microRNAs has been validated as a biomarker for development and severity of NE [503]. microRNAs have also been implicated in inflammation, including microglial activation [504], and the breakdown of the blood brain barrier in NE [505]. Recent evidence suggests that miR 17-92 family are master regulators of neurogenesis, controlling proliferation and neuronal differentiation of neural stem/progenitor cells in the developing brain [506]. In a rat pup model of hypoxia-ischaemia brain damage (HIBD), miR-20a expression was upregulated following HI injury. Dexmedetomidine decreased expression of miR-20a and resulted in reduced neuronal and hippocampal injury [507]. miR-20a has also been implicated in a mouse model of HIBD as an inhibitor of the expression of X chromosome-linked inhibitor of apoptosis (XIAP), resulting in further apoptotic cell death [508]. MiR-93 is thought to play a neuroprotective role following HI brain injury, and miR-93 levels are reduced in patients following ischaemic stroke with miR-93 levels correlated with neurological outcomes [509]. A neonatal mouse model demonstrated that HIBD was alleviated following miR-93 delivered from mesenchymal stem cell-derived extracellular vesicles [510]. MiR-582 has demonstrated neuroprotective effects following ischaemic injury by ameliorating neuronal injury through inhibition of neuronal apoptosis [511], and a



reduction in inflammation and oxidative stress [512]. Melatonin alters microRNA expression with implications for immune function and inflammation [513, 514].

## Aims

- To examine the ex-vivo effects of melatonin treatment on neutrophils and monocyte phenotype in infants with neonatal encephalopathy
- To examine the ex-vivo effects of melatonin treatment on inflammasome gene expression in infants with neonatal encephalopathy
- To examine the ex-vivo effects of melatonin treatment on circadian rhythm gene expression in infants with neonatal encephalopathy
- To examine the ex-vivo effects of melatonin treatment on microRNA expression in infants with neonatal encephalopathy

## Hypothesis

Melatonin treatment may alter innate immune responses in neonatal encephalopathy.

## Methods

### Study population

Infants were recruited from three Dublin maternity hospitals, all of which are tertiary neonatal intensive care units (NICUs) and national referral centres for TH. Infants were eligible for inclusion if they were diagnosed with moderate-severe NE, whether or not they received therapeutic hypothermia. The diagnosis of NE was based Huang criteria [459]. Written consent was obtained prior to recruitment and families received verbal and documented information on the study prior to consent. We have previously recruited several distinct cohorts of infants with NE and controls with criteria as described [43,177]. The severity of NE was classified by Sarnat staging.

### Blood Processing

Blood samples were obtained via central and peripheral arterial lines when available or venous sampling at times of routine patient phlebotomy. 1-1.4ml samples were collected in sodium citrate tubes, brought to the laboratory and processed immediately. For flow cytometry and ELISA whole blood samples were treated in 100µL aliquots, for qPCR 350µL aliquots, with lipopolysaccharide (LPS; E.coli 0111:B4: SIGMA Life Science, Wicklow, Ireland) 10 ng/mL, Pam3Cys-Ser-(Lys)4 trihydrochloride (Pam3CSK4) (TOCRIS bio-technique, Abingdon, UK) (5ng/ml), and melatonin (SIGMA Life Science, Wicklow, Ireland) (1.2µl of 42mM in 0.15% ethanol solution) alone and in combination and incubated for 1 hour at 37°C.

## Cell surface antigen expression

Flow cytometry allows analysis single cells or populations of cells in solution [515] and uses lasers as light sources which scatter light as they pass through cells in suspension. Cell size can be measured by the amount of light that is forward scattered (FSC) and cell complexity or granularity can be measured by the amount of light side scattered (SSC). Specific cells populations can be labelled using fluorescent antibodies for cell surface markers, which allows greater characterisation of specific cell populations in a mixed cell sample as well as the intensity and frequency of expression of those cell surface markers. As neutrophils and monocytes have been implicated in the pathogenesis of NE, we aimed to quantify the expression in these two cell types under different conditions of cell surface markers CD11b, a marker of cell activation, toll-like receptor (TLR)-2, a pattern recognition receptor, and NADPH oxidase 2 (NOX), a subunit of the enzyme NADPH oxidase responsible for much reactive oxygen species (ROS) production. We also aimed to quantify the frequency of intracellular cytokine expression in neutrophils of interleukin (IL)-6, IL-10, IL-17a, and tumour necrosis factor (TNF)- $\alpha$ .

100 $\mu$ l of whole blood was used for each sample, and seven samples were treated for each patient. Samples were treated with melatonin and either pro-inflammatory stimuli lipopolysaccharide (LPS) or Pam3CysSerLys4 (PamCSK). Control samples were treated with Phosphate-Buffered Saline and 0.15% ethanol. Once treated, samples were briefly vortexed and placed on a heat block to be incubated at 37°C for 1 hour. Following incubation, a mixture of fluorochrome-labelled monoclonal antibodies was added to six of the seven

samples, with the remaining sample kept as the unstained control. The antibody mixture was designed for neutrophil and monocyte characterisation and for the above cell surface markers to be quantified. It contained the following antibodies CD14 - PerCP, CD15 – PECy7, CD16 – FITC, CD66b – Pacific Blue, TLR2 – APC, and CD11b – PE (

Table **25**), which were diluted in phosphate buffered alanine (PBA). 40µl of antibody mixture was added to each of the six samples, and each was briefly vortexed before being incubated at room temperature in the dark for 15 minutes. Red cells were then lysed in each sample by adding 1ml of BD FACS™ lysing solution (BD biosciences), again incubating in the dark for 15 minutes, before centrifuging each sample at 1500RPM for 7 minutes and removing the supernatant leaving the red cells pelleted at the bottom of the Eppendorf tube. Samples were vortexed and the FACS lysis process was repeated. Using PBA, then cells were then washed twice and subsequently fixed in 500µl of 1% paraformaldehyde (PFA). To remove the PFA, samples were centrifuged at 1500RPM for 7 minutes, the supernatant removed and discarded, and the cell pellet was resuspended in 100µl of PBA and analysed on BD FACSCanto™ II flow cytometer.

For intracellular cytokine analysis the above methodology was followed with the following steps modified. Cells were stained with extracellular cell markers, lysed, and washed using the methodology above, but fixed using 4% PFA for 10 minutes. Extracellular markers included CD14 – PerCP, CD 16 – FITC, CD49a – APC-Cy7, and CD66b – Pacific Blue (

Table **26**). Once fixed, cells were incubated in the dark for 10 minutes with 500µl of saponin to permeabilize the cell membranes. Samples were then centrifuged at 1500RPM

for 5 minutes, the supernatant removed and discarded. The mixture of intracellular fluorochrome-labelled monoclonal antibodies was then added to each sample, except for the unstained control (

Table 27), and cells were stained in the dark for 20 minutes. Following this cells were washed with PBS, centrifuged, and the supernatant removed and discarded. Cells were fixed again with 1% PFA, rewashed with PBS, supernatant removed and the pellet suspended in 100µl of PBA and analysed on BD FACSCanto™ II flow cytometer.

Before starting the analysis, the FACSCanto II was set up as per standardised protocol, and the compensation set up performed with compensation controls to ensure no spectral overlap of the fluorophores. Once the compensation was set up each sample was acquired with a minimum of 50,000 events recorded and the data was exported on FCS files for analysis on FlowJo v10 software (FlowJo LLC, Oregon USA).

Cell populations were identified initially based on their FSC and SSC, dead cells were excluded, and doublets were removed (Figure 81). Using the gating strategy by Prabhu et al. cell populations were further characterised based on their cell surface marker expression [516]. Based on the expression of CD66b, positive in granulocytes, neutrophils and monocytes were distinguished. CD14 and CD16 expression was used to distinguish between classical (CD14+/CD16-), intermediate (CD14+/CD16+) and non-classical (CD14dim/CD16+) monocytes. Once cell populations of interest were identified, fluorescence intensity of other cell surface markers including CD11b, NOX, and TLR2 were quantified by mean fluorescence

intensity of all cells identified, a measure of the relative number of receptors present on the cell surface of each cell.

Antibody	Tag	Manufacturer	Volume per 100µl sample
<b>CD14</b>	PerCP	BioLegend	2.5µl
<b>CD15</b>	PECy7	BioLegend	2.5µl
<b>CD16</b>	APC-Cy7	BioLegend	2.5µl
<b>CD66b</b>	Pacific Blue	BioLegend	2.5µl
<b>CD11b</b>	PE	BioLegend	10µl
<b>NOX</b>	FITC	BioLegend	2.5µl
<b>TLR2</b>	APC	BioLegend	2.5µl
<b>PBA</b>			27.5µl

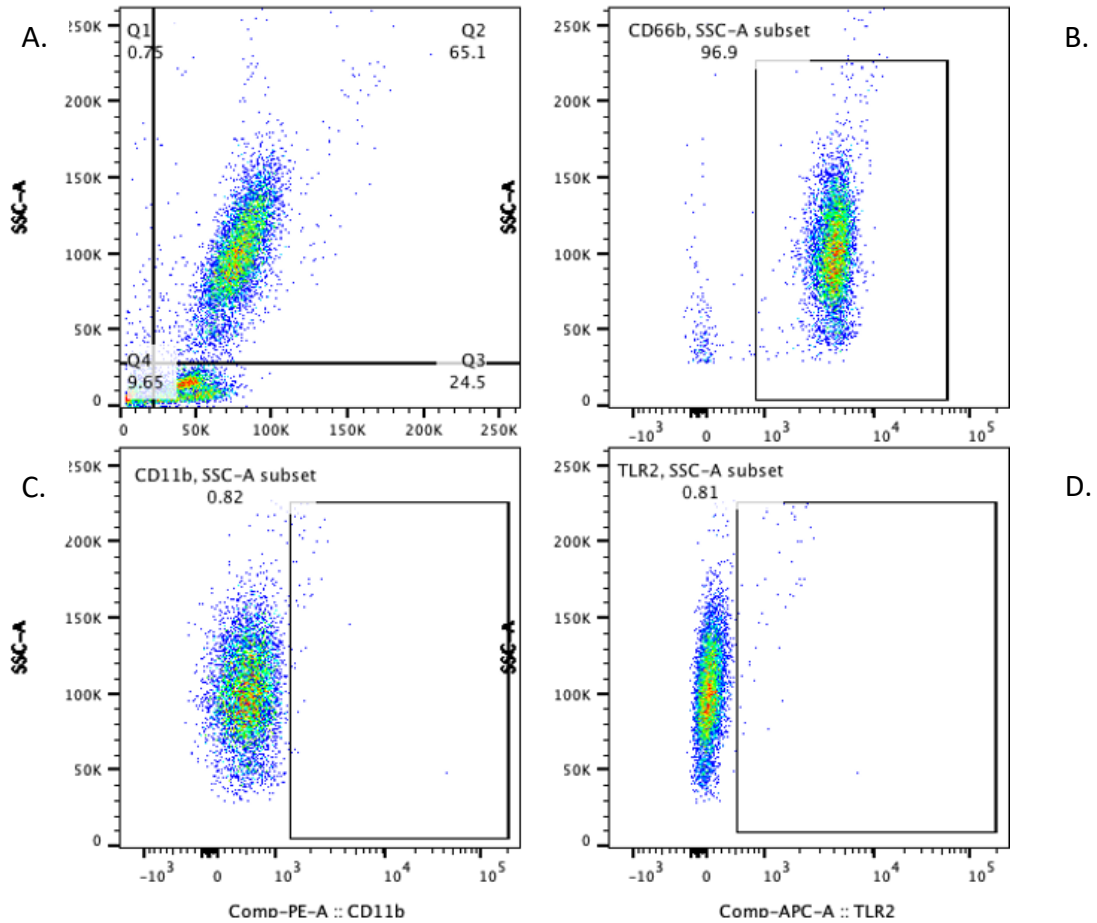
*Table 25 Antibody mixture for the study of neutrophils and monocytes markets CD11b, NOX, and TLR2.*

Antibody	Tag	Manufacturer	Volume per 100µl sample
<b>CD14</b>	PerCP	BioLegend	2µl
<b>CD16</b>	FITC	BioLegend	2µl
<b>CD49d</b>	APC-Cy7	BioLegend	2µl
<b>CD66b</b>	Pacific Blue	BioLegend	2µl
<b>PBA</b>			42µl

*Table 26 Extracellular antibody mixture for the study of neutrophils intracellular cytokines IL-6, IL-10, IL-17a, and TNF-α.*

Antibody	Tag	Manufacturer	Volume per 100µl sample
<b>IL-6</b>	PE-Cy7	BioLegend	2µl
<b>IL-10</b>	PE	BioLegend	2µl
<b>IL-17a</b>	APC	BioLegend	2µl
<b>TNF-α</b>	Amcyan	BioLegend	2µl
<b>Saponin</b>			42µl

*Table 27 Intracellular antibody mixture for the study of neutrophils intracellular cytokines IL-6, IL-10, IL-17a, and TNF-α.*



**Figure 81** Example of flow cytometry gating strategy for the identification of granulocytes based on their size and granularity using forward scatter (FSC) and side scatter (SSC) (A) and based on CD66b antigen expression (B), and the expression of cell surface antigens of interest CD11b (C) and TLR-2 (D).



## mRNA and microRNA gene expression

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is a laboratory technique that allows quantification of messenger RNA (mRNA) gene expression by first transcribing RNA to complementary DNA (cDNA), and then using cDNA as a template for quantitative polymerase chain reaction (qPCR). Following incubation with immunomodulators, 350µL samples were added to 1ml of RNAlater and stored at -80°C for later processing and analysis.

Ribopure blood kit (Thermo Fisher Scientific, Waltham, MA, USA) was used and the manufacturer's instructions followed to extract RNA from each sample. Samples were centrifuged and the resulting supernatant removed. The cell pellet was then suspended in lysis solution (800µL) and sodium acetate (50µL). Acid-phenol:chlorophorm (500µL) was then added to the cell lysate and the samples vortexed thoroughly. Following this samples were centrifuged to separate the lower (organic) phase and upper (aqueous) phase. The aqueous phase, containing the RNA, was then removed, providing approximately 1mL of RNA solution. 100% ethanol (500µL) was added to samples, and the solution was applied to a filter cartridge. Flow through from the cartridge was discarded, leaving the RNA trapped in the filter. Three rounds of Wash Solution (700µL) was added to the filter cartridge. Following this, the filter cartridge was placed in a clean collection tube and elution solution (500µL, preheated to 75°C) added to the filter paper to recover the RNA. The purity and concentration of RNA was determined using the NanoDrop ND-100 spectrophotometer and analysed using ND-1000 version 3.1.2 software. The concentration of each sample is

calculated using the ratio of measured absorbance at 260nm and 280nm. A ratio of  $\geq 1.6$  for RNA suspended in water was considered the acceptable.

Following RNA extraction and quantification, cDNA was synthesised using the High Capacity cDNA Archive Kit (Applied Biosystems) and following the manufacturer's instructions. 1 $\mu$ g of RNA was reverse transcribed to single-stranded cDNA and amplified. A 2X mastermix solution containing buffer solution, dNTPs, RT random primers, Multiscribe reverse transcriptase, and RNase-free water was prepared and added to each sample (20 $\mu$ L). Separate no-amplification control (NAC) and no-template controls were prepared simultaneously with the rest of the samples being examined. The samples were added to the thermocycler (10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and hold at 4°C) to complete the three process steps of PCR – denaturation, annealing, and extension.

Gene expression was evaluated by qPCR performed using TaqMan<sup>®</sup> RT– PCR primer probes for inflammasome genes (NLRP3, ASC and IL-1 $\beta$ ) and circadian rhythm genes (BMAL, CLOCK, CRY, and REV-ERB $\alpha$ ). Real time (RT) PCR is a technique used to measure gene expression during which the gene of interest is amplified in real time during the PCR process through use of a gene-specific fluorescent reporter. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for data normalization. All samples were assayed in triplicate. Relative quantification (RQ) values for the expression of each gene were calculated using the  $2^{-\Delta\Delta C_t}$  method [517].

## Cytokine analysis

Following incubation with immunomodulators, samples were centrifuged (10 minutes at 4°C at 1500rpm) following which the plasma was collected and stored at -80°C for later batch processing. Customised serum plates for our study and selected cytokines made by MesoScale Discovery ([www.meso-scale.com](http://www.meso-scale.com)) were used for cytokine analysis by sandwich enzyme linked immunosorbent assay (ELISA). The plates were analysed on the Sector Imager and validated. The 13 cytokines analysed were erythropoietin (Epo), granulocyte and Granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-2, IL-6, IL-8, IL-10, IL-18, interferon (IFN)- $\gamma$ , tumour necrosis factor (TNF)- $\alpha$ , TNF- $\beta$ , and vascular endothelial growth factor (VEGF). Each plate was analysed on an MSD instrument and the results were displayed in pg/ml [177].

## Statistical analysis

Data were analysed for normal distribution by visual inspection of histograms and by Shapiro-Wilk test. If the data was not normally distributed outliers were further examined for clinical or methodological variability. If outliers could not be explained they were included and we used non-parametric tests or a log transformation was applied and parametric tests were used for transformed data. Between-group differences were examined using non-parametric tests including the Wilcoxon signed rank test, Kruskal-Wallis one-way ANOVA, and the Friedman test. All tests were 2-tailed, the level of significance was set at  $p < 0.05$ , and a Bonferroni correction was applied for multiple tests. For results of flow cytometry the following between-group differences were examined: vehicle vs LPS, vehicle

vs melatonin, melatonin vs LPS & melatonin, vehicle vs PamCSK, PamCSK vs PamCSK & Mel. For qPCR and ELISA results the following between group differences were examined; vehicle vs LPS, vehicle vs melatonin, melatonin vs LPS & melatonin. For cytokine results, we explored whether there was any correlation between circadian rhythm gene expression in untreated vehicle samples and inflammatory cytokine levels in untreated vehicle samples infants with NE, given that specific CR targeted therapies are currently being explored for therapeutic use. Results were matched by patient but not by sample. We used log-transformed for both CR gene expression and cytokine levels, and the parametric test, Pearson correlation coefficient, to examine the relationship. Tests were 2-tailed and the level of significance was set at  $p < 0.05$ , and a Bonferroni correction was applied given that 13 cytokines were being explored, giving a Bonferroni-corrected significance level of 0.0038.

## Results

### Infant Characteristics

Fifty-four infants were recruited to this study including NE (n=36) and healthy neonatal controls (n=18). All infants with NE were treated with therapeutic hypothermia (Table 28). The median gestational age (GA) at birth for infants with NE was 38 weeks compared to mean GA of 40+3 weeks at birth for healthy controls. The median birthweight for those with NE was 3200gms, compared to 3900gms for healthy controls. The same proportion of participants were female in both groups, at 33%. A higher proportion of infants with NE (50%) were born by lower segment caesarean section compared to healthy controls (22%). Compared to healthy controls, infants with NE had lower Apgar scores at 1 minute, median 9 and 1 respectively, and at 5 minutes, median 9 and 4 respectively. The majority of infants with NE were intubated (92%) however only a small proportion required resuscitation with cardiopulmonary resuscitation (8%). All infants with NE underwent therapeutic hypothermia (TH) and the majority of patients had seizures prior to discharge from hospital (70%). Twenty of the surviving infants (n=33) had a normal MRI brain (60%).

	NE (n = 36)	Control (n = 18)
Gestational Age in weeks (median, IQR)	38 (38 -40)	40+3 (38 – 41)
Birthweight in grams (median, IQR)	3200 (2820 – 3630)	3900 (3400 – 4130)
Gender (female %)	12 (33)	6 (33)
LSCS (%)	18 (50)	4 (22)
Apgar 1 minute (median, IQR)	1 (1 – 3.5)	9 (8-9)
Apgar 5 minute (median, IQR)	4 (2 - 6)	9 (9)
CPR (%)	3 (8)	N/A
Intubated (%)	33 (92)	N/A
Cord arterial pH	7.09 (6.9 – 7.2)	N/A
Cord arterial BE	8.9 (7.5 – 16.7)	N/A
Therapeutic hypothermia (%)	36 (100)	N/A
Seizures (%)	25 (70)	N/A
MRI abnormal (%)	20 (60)	N/A

**Table 28 Participant characteristics for those born with neonatal encephalopathy (NE) and healthy controls.** Results are presented as median and interquartile range, or as percentages. IQR – interquartile range; LSCS – lower segment caesarean section; BE – base excess.

## The ex-vivo effects of melatonin treatment on neutrophil and monocyte CD11b, NOX, and TLR2 expression in neonatal encephalopathy

The results of the distribution of neutrophil and monocyte extracellular antigen CD11b (n = 12), NOX (n = 6), and TLR-2 (n = 10) in untreated vehicle, endotoxin (lipopolysaccharide, LPS), melatonin, endotoxin & melatonin, Pam3Cys-Ser-(Lys)4 (PamCSK), and PamCSK & melatonin treated samples were not normally distributed in infants with neonatal encephalopathy so non-parametric tests were used to examine between-group differences.

LPS stimulation resulted in an increase in CD11b expression in neutrophils compared to vehicle samples ( $p < 0.001$ ), with vehicle median MFI 18387 (interquartile range (IQR) 15322 to 22702) compared to LPS median MFI 37528 (IQR 30682 to 51330). However, there was no evidence of a difference in CD11b expression between vehicle and melatonin groups ( $p = 0.059$ ), LPS and LPS & melatonin groups ( $p = 0.45$ ), vehicle and PamCSK groups ( $p = 0.13$ ), nor PamCSK and PamCSK & melatonin groups ( $p = 1.0$ ) (Figure 82A). There was no difference in CD11b expression in monocytes between vehicle and LPS groups ( $p = 0.88$ ), vehicle and melatonin groups ( $p = 0.16$ ), LPS and LPS & melatonin groups ( $p = 0.50$ ), vehicle and PamCSK groups (0.59), nor PamCSK and PamCSK & melatonin groups ( $p = 1.0$ ) (Figure 82D).

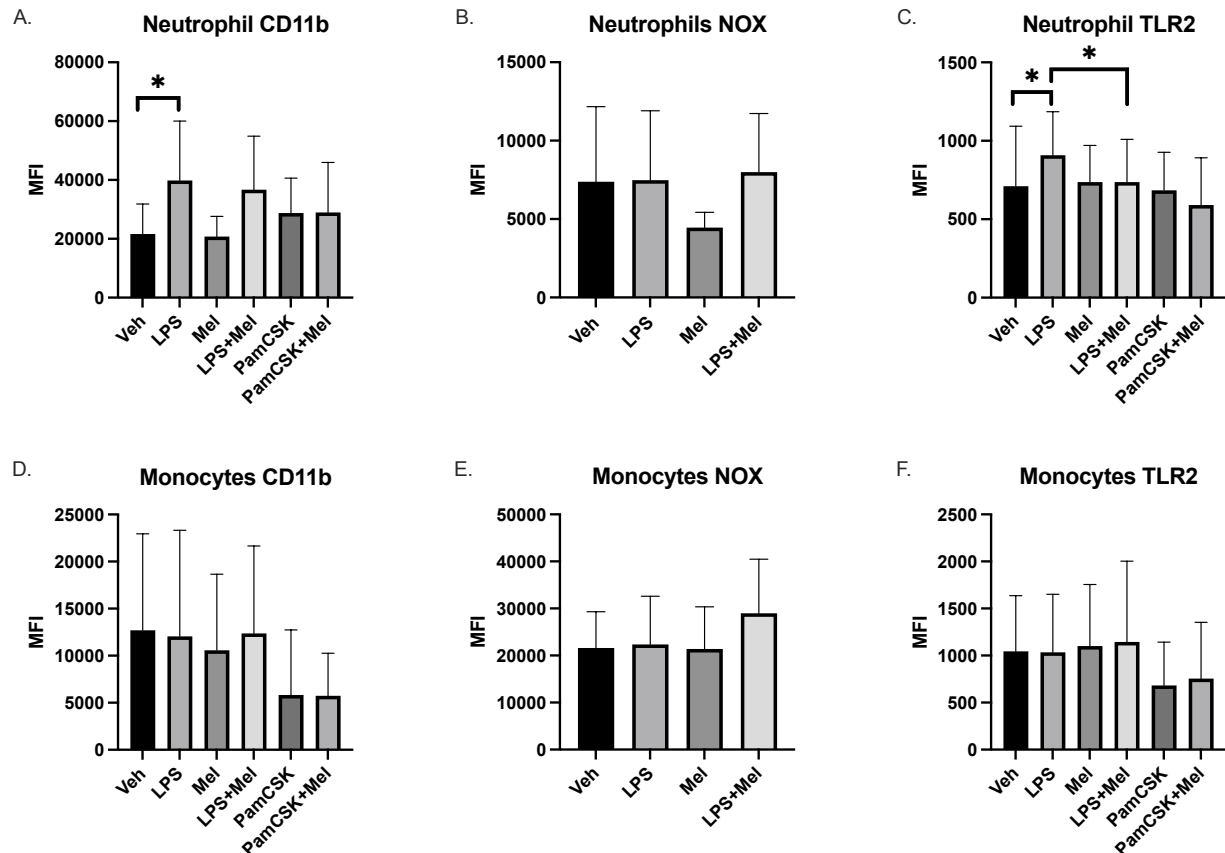
There was no difference in NOX expression in neutrophils between vehicle and LPS groups ( $p = 0.18$ ), vehicle and melatonin groups ( $p = 0.18$ ), nor LPS and LPS & melatonin groups ( $p = 0.18$ ) (Figure 82B). There was no evidence of a difference in NOX expression in

monocytes between vehicle and LPS groups ( $p = 0.72$ ), vehicle and melatonin groups ( $p = 0.18$ ), nor LPS and LPS & melatonin groups ( $p = 0.18$ ) (Figure 82E).

LPS stimulation resulted in an increase in TLR2 expression in neutrophils compared to vehicle samples ( $p = 0.011$ ), with vehicle median MFI 422 (interquartile range (IQR) 204 to 727) compared to LPS median MFI 848 (IQR 649 to 987). Melatonin treatment in LPS stimulated neutrophils resulted in an decrease in TLR2 expression in neutrophils compared to LPS stimulated samples ( $p = 0.05$ ), with LPS & melatonin median MFI 597 (IQR 446 to 802) compared to LPS median MFI 848 (IQR 649 to 987). However, there was no evidence of a difference in TLR2 expression between vehicle and melatonin groups ( $p = 0.86$ ), vehicle and PamCSK groups ( $p = 0.07$ ), nor PamCSK and PamCSK & melatonin groups ( $p = 0.14$ ) (Figure 82C).

There was no evidence of a difference in TLR2 expression in monocytes between vehicle and LPS groups ( $p = 0.89$ ), vehicle and melatonin groups ( $p = 0.58$ ), LPS and LPS & melatonin groups ( $p = 0.24$ ), vehicle and PamCSK groups ( $p = 0.59$ ), nor PamCSK and PamCSK & melatonin groups ( $p = 1.0$ ) (Figure 82F).





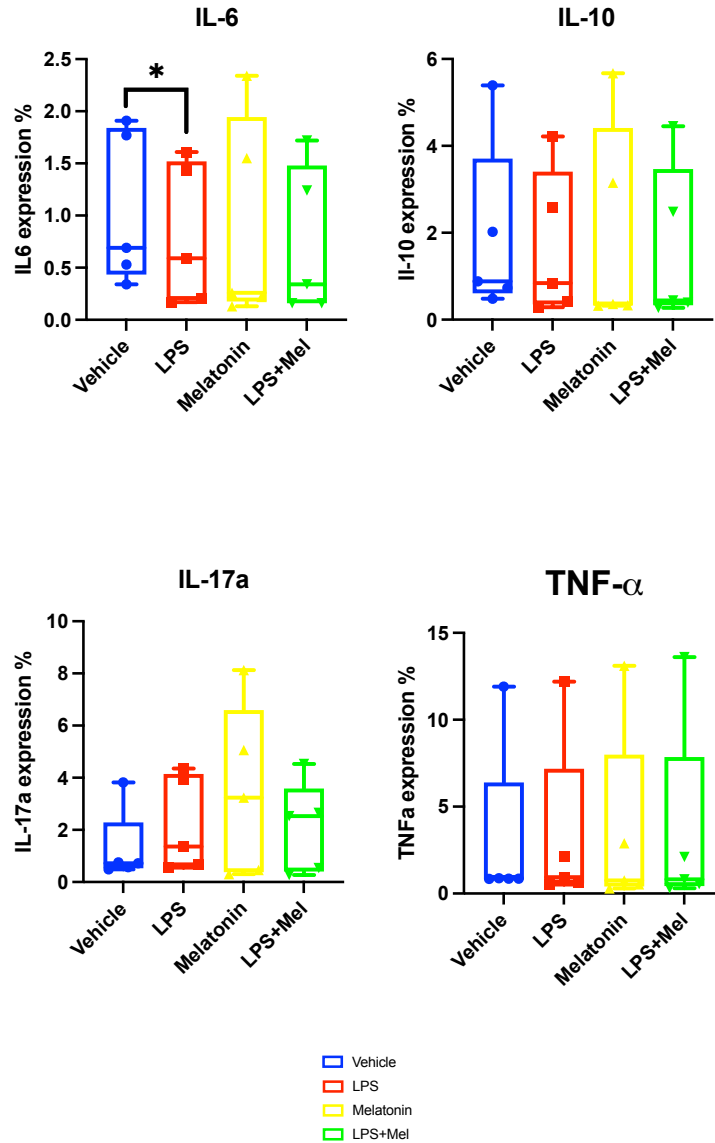
**Figure 82 Neutrophil and monocyte CD11b, NOX, and TLR2 expression in infants with neonatal encephalopathy.** Whole blood samples from infants with NE was left unstimulated (Veh), or stimulated with either lipopolysaccharide (LPS) or Pam3CSK4 (PamCSK), and treated with melatonin. Leukocyte markers CD11b, NADPH oxidase (NOX), and toll-like receptor (TLR)-2 were analysed by flow cytometry. The graph displays the mean fluorescence intensity (MFI) of CD11b, TLR-2, and NOX on neutrophils and monocytes. Neutrophils and monocytes were identified based on their size and granularity (FSC, forward scatter; and SSC, side scatter, respectively), followed by labelling with CD66b+ for neutrophils, and CD66b- and CD14+ for monocytes. Data was not normally distributed, and between group differences were analysed using the non-parametric paired-sample Wilcoxon signed rank test. Tests were 2-tailed and the level of significance was set at  $p < 0.05$ , and a Bonferroni correction for multiple tests was applied. Significant group differences are identified by \*. CD11b was increased in neutrophils in LPS-stimulated samples compared to unstimulated samples ( $p < 0.001$ ) (A). Neutrophil TLR-2 expression was increased in LPS-stimulated samples compared to unstimulated samples ( $p = 0.011$ ) and neutrophil TLR-2 expression was decreased in LPS & melatonin-treated samples compared to endotoxin (C).

## The ex-vivo effects of melatonin treatment on neutrophil intracellular cytokine expression in neonatal encephalopathy

The results of the distribution of neutrophils positive for the intracellular cytokine interleukin 6 (IL-6), IL-10, IL-17a, and tumour-necrosis factor (TNF)- $\alpha$  in vehicle, endotoxin, melatonin, and melatonin & endotoxin treated samples were not normally distributed in infants with neonatal encephalopathy (n = 5). Therefore between-group differences were examined using non-parametric tests.

There a significant reduction (p = 0.043) in IL-6 positive neutrophils following LPS (median 0.59, interquartile range 0.19 to 1.52) compared to vehicle samples (median 0.69, interquartile range 0.44 to 1.84) (Figure 83A).

Melatonin treatment did not result in any change in IL-6 (Figure 83A), IL-10 (Figure 83B), IL-17a (Figure 83C), nor TNF- $\alpha$  (Figure 83D) positive neutrophils compared to vehicle samples. Nor did melatonin result in any difference between treatment groups melatonin and melatonin & endotoxin groups in IL-6, IL-10, IL-17a, nor TNF- $\alpha$  positive neutrophils. LPS treatment did not result in any change in IL-10, IL-17a, nor TNF- $\alpha$  positive neutrophils relative to vehicle samples .



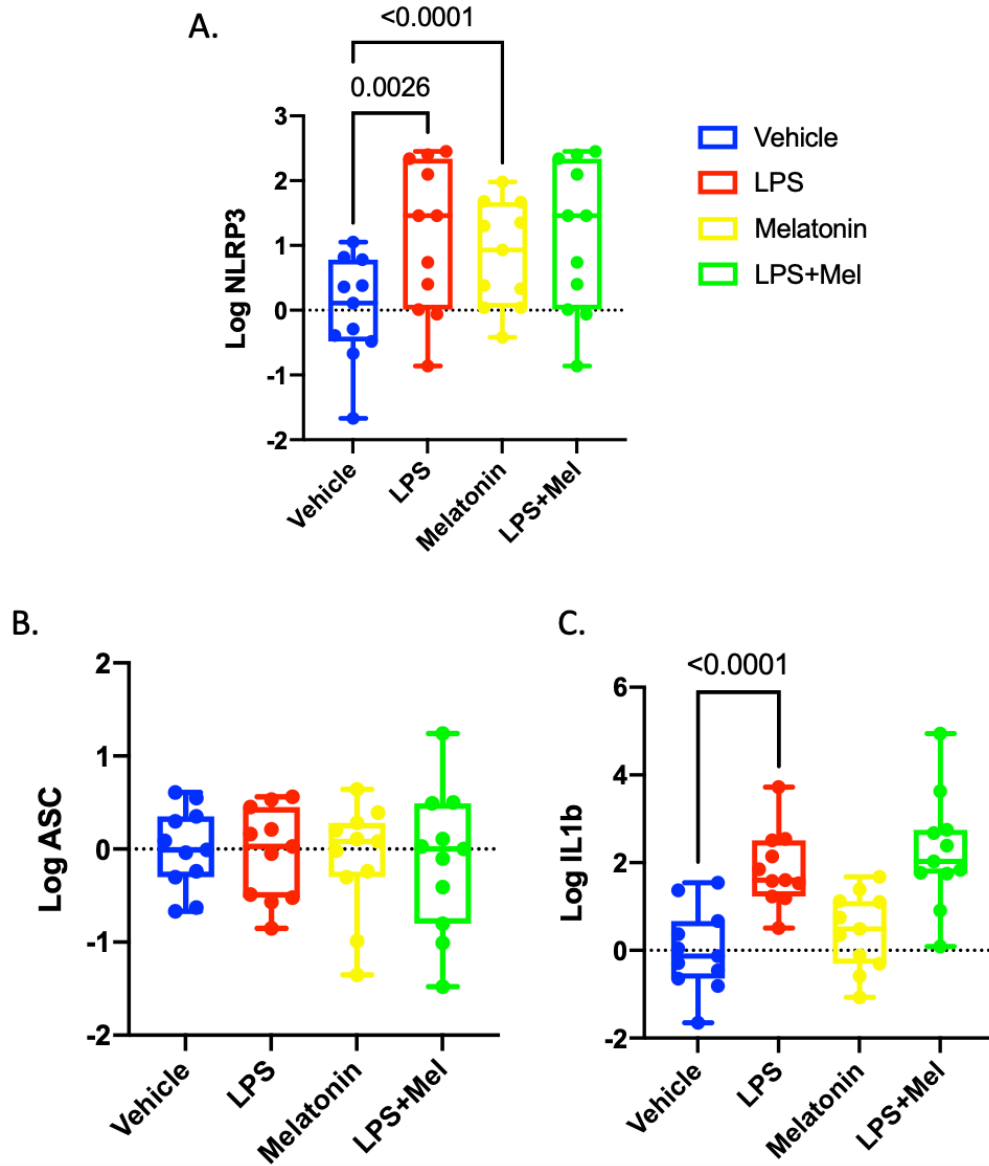
**Figure 83** Effect of endotoxin-stimulation (LPS) melatonin treatment (melatonin), and LPS& Mel on neutrophils positive for intracellular cytokines in infants with neonatal encephalopathy ( $n = 5$ ) compared to untreated control samples (Vehicle) for cytokines interleukin (IL)-6 (A) IL-10 (B) IL-17a (C) and tumour necrosis factor (TNF)- $\alpha$  (D). Values displayed represent medians and interquartile range for % change in proportion of neutrophil positive cells. Significant group differences are identified by \*. IL-6 was decreased in neutrophils in LPS-stimulated samples compared to unstimulated samples ( $p = 0.043$ ) (A). Melatonin treatment did not result in any change in IL-6 ( $p = 0.42$ ), IL-10 ( $p = 0.89$ ), IL-17a ( $p = 0.23$ ), nor TNF- $\alpha$  ( $p = 0.69$ ) positive neutrophils compared to vehicle samples. Nor did melatonin result in any difference between treatment groups melatonin and melatonin & endotoxin groups in IL-6 ( $p = 0.23$ ), IL-10 ( $p = 0.50$ ), IL-17a ( $p = 0.14$ ), nor TNF- $\alpha$  ( $p = 0.50$ ) positive neutrophils. LPS treatment did not result in any change in IL-10 ( $p = 0.35$ ), IL-17a ( $p = 0.35$ ), nor TNF- $\alpha$  ( $p = 0.69$ ) positive neutrophils relative to vehicle samples.

## The ex-vivo effects of melatonin treatment on the expression of NLRP3-inflammasome genes in neonatal encephalopathy

The results of the distribution of NLRP3, ASC, and IL-1 $\beta$  fold changes in vehicle, endotoxin, melatonin, and melatonin & endotoxin treated samples were not normally distributed. Therefore a log transformation was applied to the data and the parametric paired t-test was employed on transformed data.

Melatonin treatment resulted in increased fold change of NLRP3 expression relative to vehicle samples of log 1.13 (95% CI 0.50 to 1.77), however the difference between treatment groups melatonin and melatonin & endotoxin was not significant (95% CI -0.86 to 0.28). LPS treatment resulted in increased NLRP3 expression relative to vehicle samples (Figure 84A).

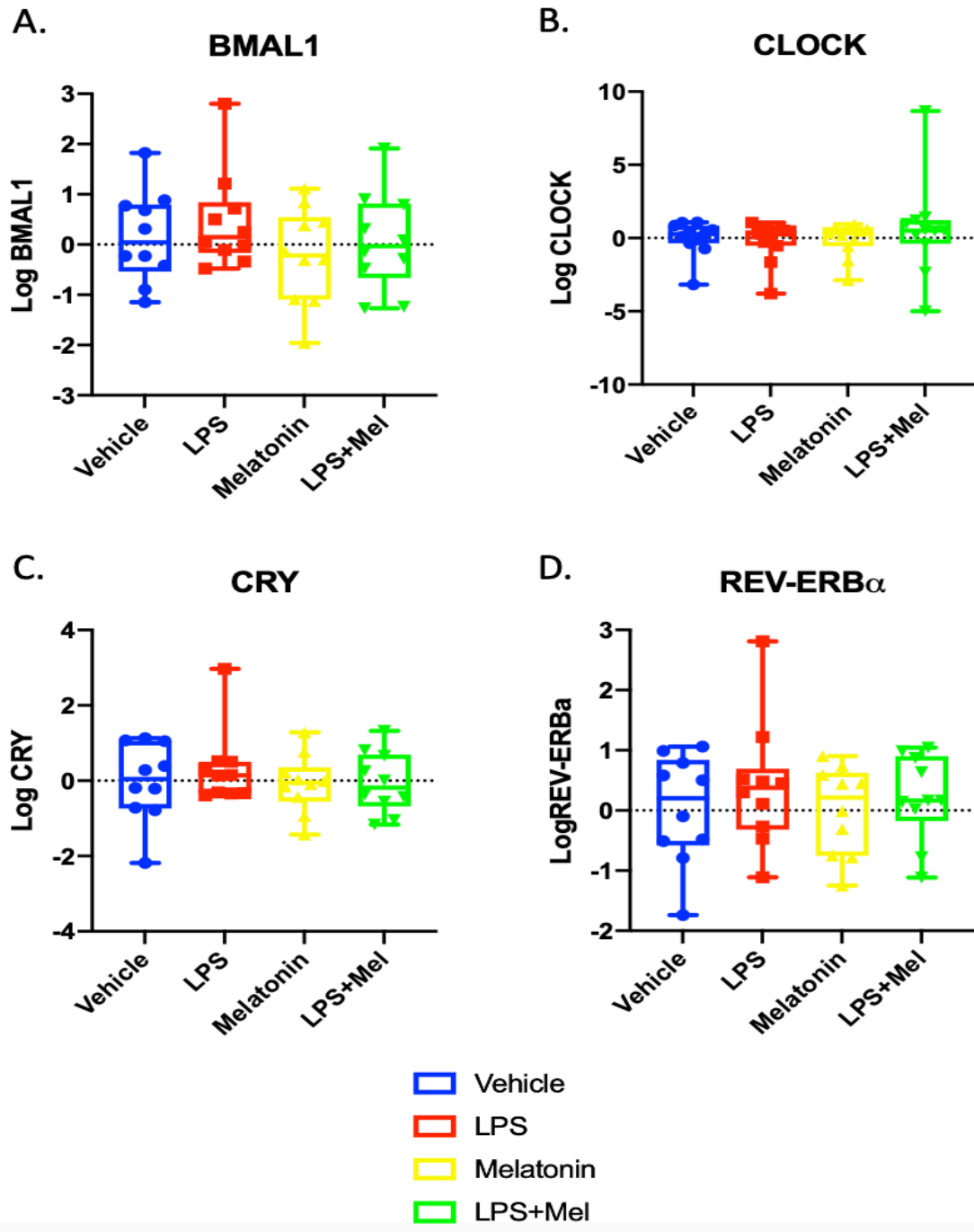
Melatonin treatment did not result in any difference in fold change of ASC (Figure 84B), nor IL-1 $\beta$  (Figure 84C) expression relative to vehicle samples, nor in any difference between treatment groups melatonin and melatonin & endotoxin in ASC or IL-1 $\beta$ . Endotoxin treatment resulted in increased fold change of IL-1 $\beta$  expression relative to vehicle samples of log 1.85 (95% CI 1.07 to 2.64) (Figure 84C) but not ASC expression (Figure 84B).



**Figure 84** Changes in mRNA expression in infants with neonatal encephalopathy ( $n = 11$ ) following endotoxin (LPS), melatonin (Melatonin), both endotoxin and melatonin treatment (LPS+Mel) compared to untreated control samples (Vehicle) for inflammasome genes NLRP3 (A) ASC (B) and IL-1 $\beta$  (C). Values displayed represent means and standard deviation fold change expression for log-transformed data. Melatonin treatment resulted in increased fold change of NLRP3 expression relative to vehicle samples of log 1.13 ( $p < 0.001$ ), however the difference between treatment groups melatonin and melatonin & endotoxin was not significant ( $p = 0.43$ ). LPS treatment resulted in increased NLRP3 expression relative to vehicle samples ( $p = 0.0026$ ) (Figure 84A). Melatonin treatment did not result in any difference in fold change of ASC ( $p = 0.38$ ) (Figure 84B), nor IL-1 $\beta$  ( $p = 0.28$ ) (Figure 84C) expression relative to vehicle samples, nor in any difference between treatment groups melatonin and melatonin & endotoxin in ASC ( $p = 0.65$ ) nor IL-1 $\beta$  ( $p = 0.22$ ). Endotoxin treatment resulted in increased fold change of IL-1 $\beta$  expression relative to vehicle samples of log 1.85 ( $p < 0.0001$ ) (Figure 84C) but not ASC expression ( $p = 0.46$ ) (Figure 84B).

## The ex-vivo effects of melatonin treatment on the expression of circadian rhythm genes in neonatal encephalopathy

The results of the distribution of BMAL1, CLOCK, CRY, and REV-ERB $\alpha$  fold changes in vehicle, endotoxin, melatonin, and melatonin & endotoxin treated samples were not normally distributed. Therefore a log transformation was applied to the data and the parametric paired t-test was employed on transformed data. There was no difference in fold change of BMAL1 (Figure 85A), CLOCK (Figure 85B), CRY (Figure 85C), or REV-ERB $\alpha$  (Figure 85D) with melatonin relative to vehicle samples, nor in any difference between treatment groups melatonin and melatonin plus endotoxin. Endotoxin treatment did not result in any change in BMAL1, CLOCK, CRY, or REV-ERB $\alpha$  expression relative to vehicle samples.



*Figure 85 Changes in mRNA expression in infants with neonatal encephalopathy (n = 11) following endotoxin (LPS) melatonin (Melatonin), both endotoxin and melatonin treatment (LPS+Mel) compared to untreated control samples (Vehicle) for circadian rhythm genes BMAL1 (A) CLOCK (B) CRY (C) and REV-ERB $\alpha$  (D). Values displayed represent means and standard deviation fold change expression for log-transformed data. There was no significant difference between treatment groups in circadian rhythm gene expression.*

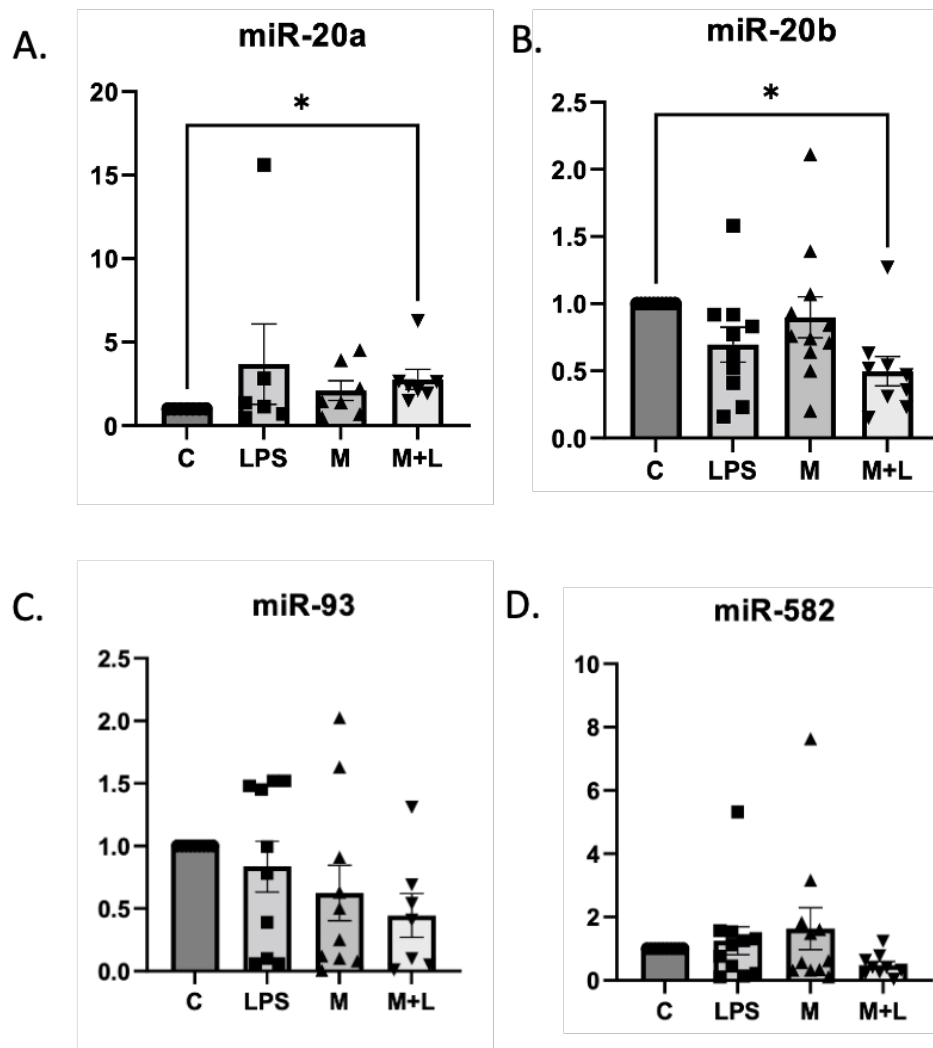
Although melatonin treatment did not result in any change in CR gene expression, we did find circadian variation in cytokine levels and circadian variation in the cytokine response to melatonin treatment in infants with NE (see Chapter 6). Inflammatory cytokines explored included IL-1 $\beta$ , IL-18, IL-1RA, IL-2, IL-6, IL-8, IL-10, EPO, GM-CSF, IFN $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , and VEGF. While some associations were significant at the level  $p < 0.05$ , none met the level of significance  $p < 0.0036$ .

#### The ex-vivo effects of melatonin treatment on microRNA expression in neonatal encephalopathy

Endotoxin & melatonin treatment resulted in an increase in miR-20a expression (Figure 86A) with a mean increase of 1.78 fold changes (95% CI 0.31 to 3.25) and a decrease in miR-20b expression (Figure 86B) with a mean decrease of 0.5 fold changes (95% CI 0.085 to 0.92) compared to control untreated samples ( $n = 11$ ). However there was no evidence that endotoxin treatment or melatonin treatment alone significantly changed miR-20a nor miR-20b expression compared to control samples.

There was no evidence that endotoxin treatment alone, melatonin treatment alone, nor the combination of endotoxin and melatonin treatment resulted in any change in miR-93 (Figure 86C) nor miR-582 (Figure 86D) expression compared to control samples ( $n = 11$ ). We examined miR-155 in the same patient cohort during the same batch as the above results. However, there was no amplification in any of the samples or any condition which is surprising given the stimulation with LPS endotoxin.





**Figure 86** Changes in microRNA expression in infants with neonatal encephalopathy ( $n = 11$ ) following endotoxin (LPS) melatonin (M), both endotoxin and melatonin treatment (M+L) compared to untreated control samples (C) for microRNAs miR-20a (A) miR-20b (B) miR-93 (C) and miR-582 (D). Values displayed represent medians and range for fold change expression. Endotoxin & melatonin treatment resulted in an increase in miR-20a expression ( $p = 0.025$ ) (Figure 14A) and a decrease in miR-20b expression ( $p = 0.015$ ) (Figure 14B) compared to control untreated samples ( $n = 11$ ). However there was no evidence that endotoxin treatment alone significantly changed miR-20a ( $p = 0.27$ ) nor miR-20b ( $p = 0.18$ ) expression, nor that melatonin treatment alone significantly changed miR-20a ( $p = 0.83$ ) nor miR-20b ( $p = 0.87$ ) expression compared to control samples. There was no evidence that endotoxin treatment alone ( $p = 0.85$ ), melatonin treatment alone ( $p = 0.32$ ), nor the combination of endotoxin and melatonin treatment ( $p = 0.12$ ) resulted in any change in miR-93 expression compared to control samples (Figure 86C). There was no evidence that endotoxin treatment alone ( $p = 0.97$ ), melatonin treatment alone ( $p = 0.72$ ), nor the combination of endotoxin and melatonin treatment ( $p = 0.84$ ) resulted in any change in miR-582 expression compared to control samples (Figure 86D).

## Discussion

We found that melatonin treatment resulted in few significant effects on the innate immune response in NE contrary to the expected results as melatonin is a potent immunomodulating agent. Overall, we found that melatonin treatment resulted in reduced neutrophil TLR-2 expression in endotoxin-stimulated samples only and increased NLRP3 inflammasome gene expression in whole blood, but there was no evidence that there was any significant change in other neutrophil or monocyte changes, no change in intracellular cytokines, no change in circadian rhythm gene expression, nor any significant change in miRNA expression.

Neutrophils and monocytes have been implicated in the pathogenesis of NE [43]. We found that melatonin treatment reduced neutrophil TLR-2 expression following endotoxin stimulation, but not compared to controls. TLR-2 is a PRR which acts as heterodimers with TLR-1 and TLR-6, and its activation is the initial step in a cascade of events in the innate immune response including the production of pro-inflammatory cytokines [518]. Experimental models have demonstrated upregulation of TLR-2 expression following hypoxic-ischaemic insult in neonatal mice, and TLR-2 knockout mice showed decreased infarct volume compared to wild-type mice [519]. TLR-2 has been implicated in the response to LPS [520], however Pam3CSK is the primary agonist to induce an inflammatory response for TLR-2 [521]. We found that TLR-2 expression was increased with LPS stimulation, but not with Pam3CSK4 in neutrophils, and there was no significant effect of either LPS nor Pam3CSK in monocytes. TLR-2 expression was decreased in LPS-stimulated

samples, but only in neutrophils. There is very limited experimental evidence to date that has examined the effect of melatonin treatment on TLR-2 expression following hypoxic-ischaemic injury. Experimental evidence suggests that there is a negative feedback loop of melatonin on TLR-2, as melatonin inhibits TLR-2 signalling, and TLR-2 blocks 5-HT (serotonin) converting to melatonin [522].

We found that CD11b was increased in neutrophils following LPS stimulation, in keeping with previous experiments [43], however there was no change with melatonin treatment. Expression of CD11b, a marker of neutrophil and monocyte activation, is increased in infants NE and adverse outcome [44]. Experimental models have demonstrated reduced CD11b expression with melatonin treatment [523], however this was not the case in our study. Hypoxia stimulates increased NOX expression and are responsible for ROS generation [524]. Melatonin reduces NOX expression and oxidative stress in neonates [188]. We found no significant effect of melatonin treatment on NOX expression.

We demonstrated that both melatonin treatment and endotoxin stimulation increased NLRP3 expression in infants with NE. The response of NLRP3 to melatonin treatment was surprising and contrary to previously published results [498]. NLRP3 is upregulated via hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) pathway in response to hypoxia resulting in increased production of the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 [525]. However, this relationship is more complex and less well understood in the neonatal population. NLRP3 is upregulated in response to LPS stimulation in neonates with NE on day three of life, but there is no difference on day one of life [497]. Similarly, we did not find a significant

association between either IL-1 $\beta$  nor IL-18 and an increased risk of adverse outcome in NE (See Chapter 4). Melatonin inhibits NLRP3 activity and the production of pro-inflammatory cytokines [498]. As previously described, melatonin treatment induced a complex alteration in cytokine production in NE, and some of these may cause increased NLRP3 gene expression. Nevertheless, there was no change in ASC gene expression in response to either LPS stimulation or melatonin treatment. ASC interaction with NLRP3 is required for inflammasome assembly [494]. There was no change in IL-1 $\beta$  or IL-18 concentration following melatonin treatment compared to vehicle samples or LPS-stimulated samples in infants with NE (Chapter 6). However, melatonin treatment did result in an increase in IL-1 receptor antagonist (IL-1RA), the inflammasome inhibitor (Chapter 6). One issue with the current results is that sampling took place over the first week of life. IL-1 $\beta$  has only been demonstrated to be increased in NE in the first 24 hours of life [118]. There was an insufficient sample size to assess for the influence of timing of sampling on the effect of melatonin on gene expression in this study. Another limitation is that this work was conducted on whole blood. NLRP3 expression may vary between different immune cells and cause a variety of effects. It is also unclear whether this response to melatonin treatment would be the same in neonatal controls compared to infants with NE and this requires further exploration.

We did not find any change in the expression of CR genes BMAL1, CLOCK, CRY, nor REV-ERB $\alpha$ . This was a surprising result given the prior strength of evidence that melatonin induces changes in CR gene expression. Hypoxia induces alterations in circadian rhythm (CR) gene expression through a mechanism dependent on HIF-1 $\alpha$ , as HIF-1 $\alpha$  directly binds to

BMAL1 E-box circadian genes directly altering the molecular clock [526]. Hypoxia can lead to expression of CR genes at inappropriate times [527]. This is a bidirectional relationship. The strength of response of HIF-1 $\alpha$  to hypoxic insults is clock-regulated, and gated by the molecular clock [528]. These interactions mean CR genes have a significant influence on the inflammatory and metabolic response to hypoxia [529]. Melatonin is a chronobiotic agent [77] meaning it shifts the endogenous circadian phase, and alters the expression of CR genes [79, 80]. One of the limitations of this study is that samples were taken throughout the 24 hour cycle and the sample size did not allow for examination of the response to melatonin treatment and different time points. It is also unclear whether the expression of CR genes is disrupted in infants with NE compared to the general neonatal population and a comparison of the response to melatonin treatment between these groups would be valuable to explore in future studies.

We did not find that any change in the gene expression of miR-20a, miR-20b, miR-93, miR-582 in response to melatonin treatment compared to vehicle or LPS-stimulated samples. miR-155 is a key regulator of the IL-1 family [530], however it did not amplify in this experiment, even in response to LPS stimulation, which was unexpected. The expression of several microRNA (miRNA) are altered in response to hypoxia [531], and in particular regulate hypoxia inducible factor (HIF) switch [532]. Therefore, miRNA have become therapeutic targets in acute ischaemic conditions in recent years [533]. Melatonin regulates the expression of several miRNA involved in the response to hypoxia [513, 534]. Some limitations of the study include as small sample size, n = 11, and again that the

samples were collected over the first week of life when various different stages of injury with different underlying inflammatory responses have been identified [18]. There are over 2,600 miRNA in the human genome and we selected 5 for analysis. The evidence for the role of miRNA in humans with neonatal encephalopathy is very limited to date and requires further exploration to understand their influence on inflammation and outcomes in the condition. Once this is further clarified more miRNA targets and their response to melatonin treatment should be investigated.

One of the significant limitations of this study and one potential explanation for the lack of significant measured effect of melatonin on the innate immune system may be due to a small sample size in most included experiments. For example, melatonin is a potent anti-oxidant and has been demonstrated to regulate the respiratory burst in human neutrophils [535], and NOX is a key enzyme in neutrophil ROS production [536]. However, our study found many significant changes in cytokine levels following melatonin treatment in both NE and controls suggesting that melatonin is a potent immunomodulator, increasing the likelihood that the sample size was not sufficient to capture the effects of melatonin in this study. The sample size for the ELISA experiments for cytokine measurement (n = 36) was substantially larger than for any of the flow cytometry or qPCR experiments in this study. Another potential explanation for the lack of significant effects of melatonin on the innate immune system in NE is that melatonin has demonstrated a variety of different immune regulating effects depending on the clinical context [108]. There are many different causes of NE and each may result in different immune dysregulation and may require a

different immune-modulating effect [2]. Another limitation and potential explanation for these findings is that we analysed samples across the first week of life. Several different stages of injury have been identified in NE and each is associated with different aspects of immune dysregulation [18, 537]. Another limitation in this study is the use of whole blood samples for many experiments. Much consideration was given to the possibility of single cell analysis from whole blood samples, however no satisfactory method was identified for cell isolation given the small blood volume samples obtained.

## Conclusion

Melatonin alters cytokine responses although this was not reflected in this paper. There are several limitations in this study and other explanations to explain these findings. Further research and analysis is essential to understand the immune-modulating effects of melatonin in NE, particularly in regard to the timing of melatonin treatment in NE, the role of melatonin as an anti-oxidant in NE, and the role of melatonin on changes in neutrophil and monocyte function in NE.

## Chapter 8: Sleep, inflammation, and outcomes in neonatal encephalopathy

### Abstract

**Background:** Circadian rhythms regulates the innate immune system. Both innate immune dysregulation and delayed onset of sleep wake cycling are associated with worse outcome in Neonatal Encephalopathy (NE). We hypothesised that infants with NE and delayed sleep onset or poorer sleep quality would have more dysregulated immune responses and higher rates of adverse outcome. We aimed to evaluate the association of sleep quality with measures of systemic inflammation and outcomes in NE.

**Methods:** Continuous aEEG recordings were collected on all infants undergoing therapeutic hypothermia for a minimum of 72 hours. Onset of sleep-wake cycling (SWC), defined by Takenouchi (2011), and sleep quality assessed by Burdjalov (2003) scoring system was applied to these recordings. Whole blood was collected from infants with NE during the first 2 days of life. A panel of 12 pro and anti-inflammatory cytokines were evaluated. Barkovich scoring system was applied to MRI imaging to determine adverse outcome. Statistical analysis employed independent t-tests and Spearman correlation coefficient.

**Results:** Infants with NE undergoing TH (n=44) were recruited and divided into normal and adverse outcomes based on MRI brain results. Infants with earlier onset of SWC and better sleep quality (SQ) had lower rates of abnormal MRI findings. SQ provided better prognostic value and showed better interobserver agreement compared to time of onset of SWC.



Better SQ was associated with lower levels of the inflammatory cytokines EPO and interleukin (IL)-1 $\beta$ . In infants with unfavourable outcome shorter time to onset of SWC was associated with higher EPO and better SQ was associated with lower TNF- $\alpha$ . There were no significant relationships between time to onset of SWC or SQ and cytokines IFN- $\gamma$ , IL-1 $\alpha$ , IL-1RA, IL-6, IL-8, IL-18, and VEGF.

**Discussion:** Infants with NE and either earlier onset of SWC or better sleep quality had less dysregulated systemic inflammation and were at lower risk of adverse outcome. Sleep quality during TH provided better prognostic information than time of onset of SWC. Modulation of the circadian rhythm in infants with NE may have an immunomodulatory role and lead to improved outcomes.

## Introduction

Sleep and circadian rhythms are critical regulators of the immune system and inflammation [480]. Fetal circadian rhythms in sleep, and secretion of hormones including melatonin, dopamine and glucocorticoids, and circadian gene expression are entrained to and coupled with maternal signals by 28-30 weeks of gestation [83, 538]. After birth circadian rhythms are gradually entrained over the first few months of life [85]. Newborns display sleep wake cycling as early as the first 6 hours of life [91], although it takes at least 10-12 weeks for sleep cycles to display diurnal rhythms [539].

Dysregulated inflammation is key process in brain injury in neonatal encephalopathy (NE) [30]. Sleep is a key regulator of the immune function and there is evidence of a bi-directional relationship with the innate immune system [540]. Sleep, a CNS mediated psychophysiological process, has a dynamic role in the regulation of the distribution of immune cells and the production of inflammatory cytokines [480]. Sleep exerts an effect on effector systems such as the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system to regulate the release of cortisol, a potent anti-inflammatory mediator, and to modulate the release of noradrenaline, a key modulator of the innate immune system that promotes activation of the inflammatory response. Further, cytokines such as IL-1 function as somnogens during times of inflammatory stress, promoting sleep and supporting host defence [541]. Sleep disruption has been associated with increased production of pro-inflammatory cytokines IL-6, and TNF- $\alpha$  [542], and increased activation of inflammatory transcriptional activity including NF- $\kappa$ B and AP-1 [543].

Sleep also promotes brain development by providing support for changes in brain activity, promoting glymphatic function in clearing toxins from the brain [544] and reducing DNA damage and potentially promoting DNA repair [545]. There is also evidence of sleep disruption in infants with NE, with longer duration to sleep wake cycling (SWC) being associated with adverse outcome in NE [92]. Further, while it is not fully understood what promotes sleep in the neonatal period, the most likely factors are light exposure primarily, feeding, and, less significantly, social cues [86, 90]. These are all disturbed in infants undergoing TH in NICU [90, 95, 546].

Although both sleep and inflammation responses are disrupted in NE, it remains unclear whether there is a causal relationship, and in which direction any causal relationship may be. Regardless, both are potentially modifiable if a causal relationship is established. In preterm infants cycled light has demonstrated improved outcomes compared to continuous dim lighting [93]. However, a systematic review of interventions to improve sleep in the NICU found there is insufficient evidence to recommend any particular intervention to promote sleep at present [547]. Many immunomodulatory agents are currently being investigated as adjunctive therapies in NE [548] although none is currently used in routine practice. Circadian and sleep control of the immune system may be useful in modulating dysregulated inflammation associated with brain injury in NE.

## Hypothesis

Delayed onset of sleep-wake cycling (SWC) and worse sleep quality (SQ) are associated with greater inflammatory dysregulation and adverse outcome in infants with NE. Severity of injury in NE will correlate with duration of time to SWC and with degree of dysregulation of the inflammatory response. SQ may provide greater prognostic information to predict likelihood of adverse outcome in infant with NE and better interobserver agreement than time to SWC. Both measures of sleep disruption may correlate with dysregulated inflammation in infants with NE.

## Aims

- To examine the association between time to SWC and outcome in NE, defined by death or abnormal MRI brain.
- To explore the association between SQ and adverse outcome in NE and compare the prognostic value of the 2 measures of sleep in NE to predict adverse outcome.
- To evaluate the association between the measures of sleep and inflammation, defined by concentrations of pro- and anti-inflammatory cytokines, in infants with NE.

## Methods

### Study Population

Infants with moderate-severe neonatal encephalopathy (NE) being treated with therapeutic hypothermia (TH) were prospectively recruited between August 2016 and January 2019 as part of the NIMBUS study [43, 450]. Recruitment took place across Dublin's three level three neonatal intensive care units (NICUs) which are national centres for TH and which combined have approximately 27,000 deliveries per annum. Infants were eligible for inclusion if they were diagnosed with NE and met the criteria previously published [450]. Infants with NE were classified by severity of neurological dysfunction according to Sarnat staging [288]. Infants born prematurely at <35 weeks gestation, infants with major congenital abnormalities, or infants born to mothers with a history of substance misuse were excluded.

### Sampling and cytokine analysis

Blood samples were taken from enrolled infants and collected in sodium citrate bottles during routine clinical phlebotomy during the first four days of life and brought to the laboratory for immediate processing. When available, samples were collected from umbilical or peripheral arterial lines, or otherwise by peripheral venous sampling. Samples were treated with phosphate-buffered solution (PBS) and incubated for one hour at 37°C. Cell supernatant was isolated from the whole blood samples by centrifugation and stored in the freezer at -80° until batch analysis by Enzyme Linked Immunosorbent Assay (ELISA).

Customised plates for this study and selected cytokines were prepared by MesoScale Discovery ([www.meso-scale.com](http://www.meso-scale.com)). Plates were analysed on the Sector Imager and validated. All cytokines are measured in pg/ml. Measured cytokines included EPO, GM-CSF, IFN $\gamma$ , IL-1 $\alpha$ , IL-1RA, IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-18, TNF- $\alpha$ , TNF- $\beta$  and VEGF.

#### aEEG recording and analysis

aEEG recordings were routinely started within a few hours for all infants treated with TH for NE. Data was collected from electrodes placed over the frontotemporal and parietal regions for assessment. These recordings were started prior to TH and lasted for the duration of TH. They were subsequently stored on CD discs for later review. aEEG recordings for all infants were retrospectively reviewed by 2 reviewers independently (TH and PS) who were blinded to infant outcome. Where differences in assessment arose following independent review of aEEG recordings by each reviewer, consensus was agreed following a third combined aEEG review. Time from birth to onset of aEEG recording was documented when sufficient infant data was available. aEEG recordings were reviewed from the earliest available time until the presence of sleep wake cycling (SWC) was detected, defined by Takenouchi et al as the 'presence of features of both wakefulness or active sleep and quiet sleep with at least two clear state changes during a 6-hour epoch' [549]. The aEEG recordings were then reviewed a second time and sleep quality (SQ) was scored according to the classification by Burdjalov et al [550]. This scoring system graded electrographic evidence of sleep according to the presence of continuity, the presence of cycling, amplitude of the lower border, and bandwidth span and amplitude of the lower

border. A stable 4-hour epoch was selected to be analysed using component variables of the aEEG scoring system. All variables were scored according to the individual component scores. This revealed a total sum for each recording of each individual infant. The minimal possible scoring was 0 and the maximum score was 13 (Appendix VIII B).

#### MRI acquisition and analysis

Adverse outcome was defined as death or abnormal MRI brain. Surviving infants underwent MRI within the first two weeks of life in one of two national paediatric referral centres in Ireland, Children's Hospital Ireland (CHI) at Crumlin or CHI at Temple Street, or when available in one of the primary research sites, the National Maternity Hospital. MRI images included conventional T1/T2 weighted images, diffusion weighted images, and proton magnetic resonance spectroscopy and they were reviewed and scored by an experienced consultant paediatric radiologist who was blinded to infant outcome (A.B. or G.C). Barkovich [309], NICHD [310], and de Vries [318] MRI scoring systems were applied to all available MRI images, however Barkovich scoring system was used for the definition of adverse outcome in this study as it provides excellent prognostic value for later neurodevelopmental outcome [551] and it is the most widely used and accepted scoring system at present for infants with NE (See Chapter 3). The Barkovich scoring system examines the basal ganglia or thalamus (BG/T), applying a score of 0-4, and watershed (WS) areas, applying a score of 0-5. These scores are combined to provide a summary score from 0, indicating normal MRI, to 9, indicating the most extensive injury on MRI (Appendix VIII C).

Abnormal MRI brain was classified according to Barkovich scoring system. A score  $\geq 1$  indicated an abnormal MRI brain.

### Statistics

All data was assessed for normality of distribution by visual inspection of histograms and by Kolmogorov-Smirnov. If data was not normally distributed, a log transformation was applied, or non-parametric tests were employed. Infants were categorised by outcome and group differences in time to SWC or SQ score between those with and without the primary outcome were examined. Group differences were examined using the Mann-Whitney U test. Correlation between time of onset of SWC or SQ scores, and MRI scoring systems were examined by Spearman's correlation coefficient ( $r_s$ ). All tests were 2-tailed, and the level of significance was set at  $p < 0.05$ . Receiver operating characteristic (ROC) curves were used to compare the discriminatory ability of time to SWC and SQ to predict adverse outcome in NE, and the sensitivity, specificity, positive predictive value and negative predictive value were calculated. The interobserver agreement between the assessors of time to SWC and SQ score was measured by a non-parametric test, the Kendall's concordance coefficient ( $W$ ). Correlations between different scoring systems were examined using Spearman's correlation coefficient. Statistical analysis was completed using SPSS (IBM SPSS statistics version 27).



## Ethical approval

This research was conducted as part of the NIMBUS study which was approved by the research ethics committee in each of the three included maternity hospitals (Appendix I). Parents or guardians were provided with verbal information and a written patient information leaflet prior to consent for and enrolment in the research study (Appendix II and III).

## Results

There were 44 infants included in this study. All infants had continuous brainwave activity measured by amplitude-integrated electroencephalography (aEEG) recording for >60 hours during TH as a routine part of their clinical treatment. Full outcome assessment was not possible for five infants due to missing MRI data. Two infants died prior to MRI assessment. 21 of the remaining 37 infants (57%) had abnormal MRI brain reported and 16 of 37 infants (43%) had normal MRI brain reports.

aEEG recordings were available for all 44 included infants. Mean duration of time from birth to onset of aEEG recording was 4.2 hours. SWC was detected in the recording of 43 of 44 included infants (98%). SQ score could be calculated for all included infants and ranged from 0 to 13. All but one infant achieved SWC during the time of recording. The population median time to SWC was 13.5 hours and the interquartile range (IQR) was 4 to 37.5 hours. The population median SQ score was 12 and the IQR was 9 to 13. Results of duration to onset of SWC or and SQ scores for included infants with NE were not normally distributed (Appendix VIII D and E), and this was confirmed by Kolmogorov-Smirnov tests (Appendix VIII F).

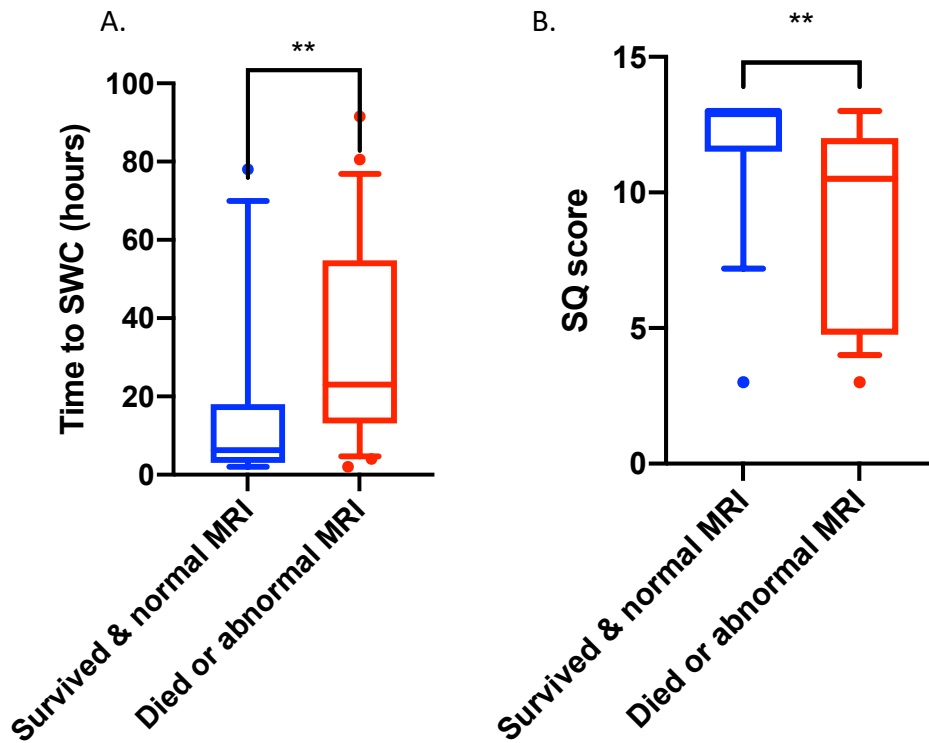
MRI results were available for 37 of the 44 included infants (84%). 16/37 (43%) infants had normal MRI brain, Barkovich score 0, and 21/37 (57%) had abnormal MRI brain, Barkovich score  $\geq 1$ . Therefore, 23/39 (59%) infants had the primary definition of adverse outcome for this study. The median Barkovich score was 2 and the IQR was 0 to 4. Of the three infants who died in this study, only one had MRI brain and it was reported as

demonstrating abnormalities. MRI Brain Barkovich scores were not normally distributed (Appendix VIII G).

#### Comparison of differences in time to SWC and SQ between groups

There was a statistically significant difference in time to SWC between groups of infants with normal and adverse outcome (Figure 87A), with infants that died or had abnormal MRI brain taking longer to achieve SWC than those that survived with normal MRI brain. For infants that survived with normal MRI brain the median time to SWC was 6 hours and the IQR was 3 to 18 hours. For infants that died or had abnormal MRI brain the median time to SWC was 23 hours and the IQR was 13 to 55 hours.

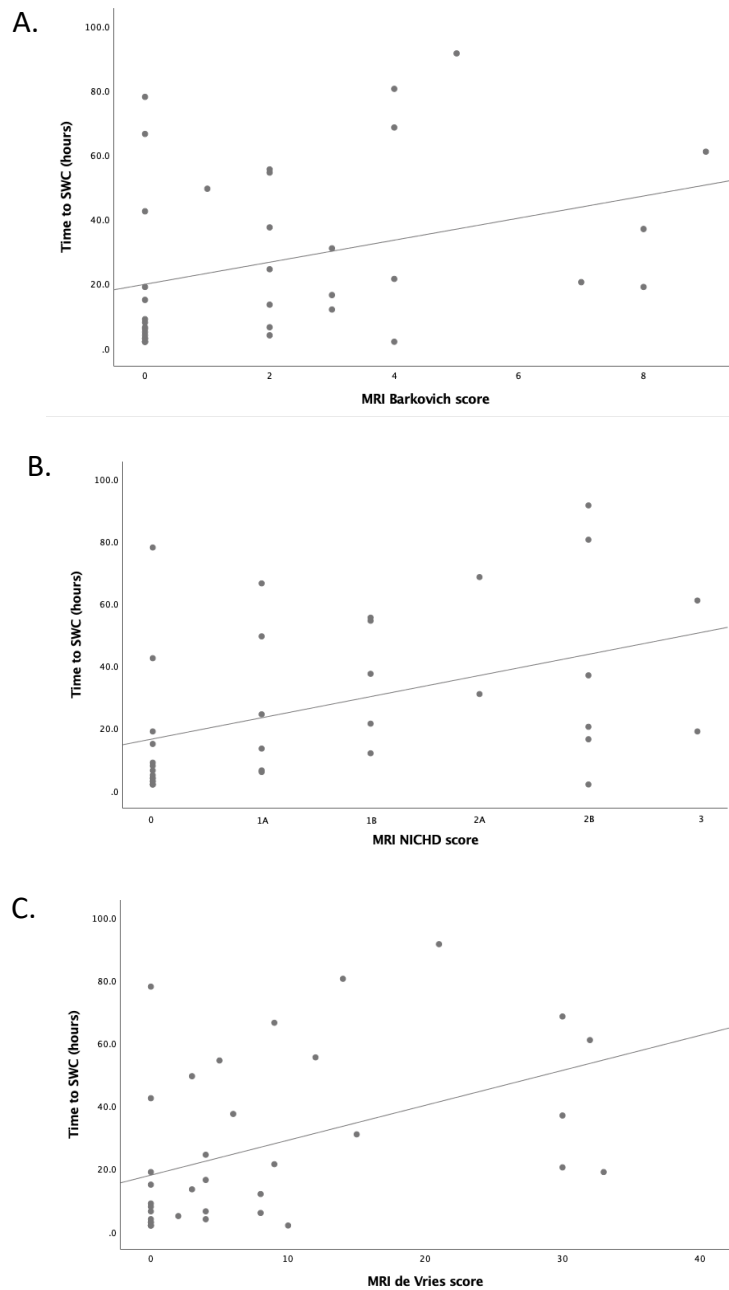
There were statistically significant differences in SQ score between groups,  $p = 0.002$  (Figure 87B), with lower SQ score in those infants who died or had abnormal MRI brain than those that survived with normal MRI brain. Infants with NE and survival with normal MRI brain the median SQ score was 13 (IQR 11.5 -13;  $p < 0.05$ ) compared to those who died or had abnormal MRI brain with a score of 10.5 (IQR 4.75 -12;  $p < 0.05$ ).



**Figure 87** Differences in time to sleep wake cycling (SWC) and sleep quality (SQ) in infants with neonatal encephalopathy and good outcome (survived & normal MRI) or adverse outcome (died or abnormal MRI) (n = 44). Differences between groups were significant in time to onset of SWC ( $p = 0.009$ ) (A) with infants with adverse outcome taking longer to achieve SWC. Differences between groups were significant in SQ score ( $p = 0.002$ ) (B) with infants with adverse outcome having lower SQ score.

## Correlation of time to SWC and MRI scoring systems

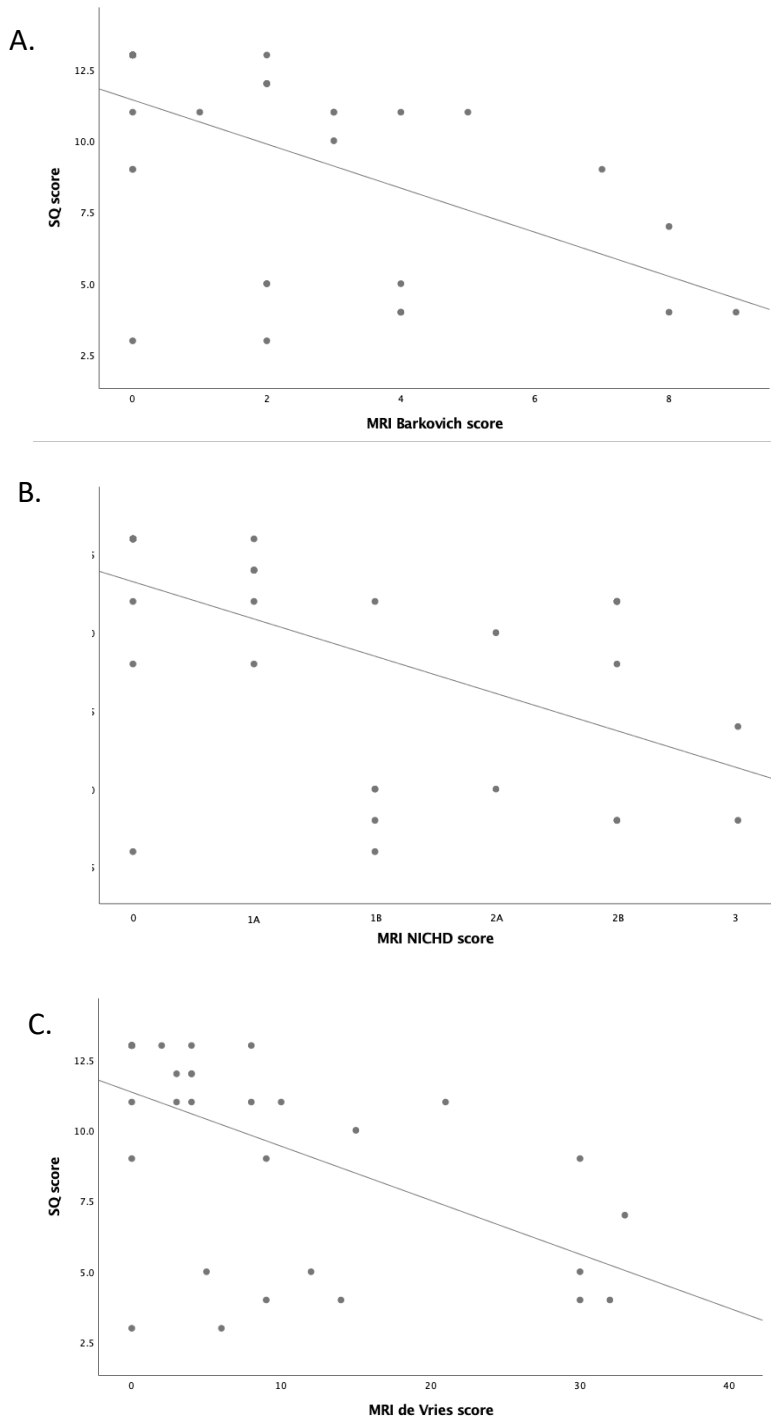
Statistically, there was a significant positive correlation between longer time to onset of SWC and higher Barkovich (Figure 88A), NICHD (Figure 88B), and de Vries scores (Figure 88C). These all indicated that longer time to onset of SWC is correlated with greater abnormalities on MRI brain in NE. However, it is clear from (Figure 88A-C) that there is very wide variation in MRI results, and that there is not a predictable relationship between these variables. Therefore, although these findings suggest that there may be an overall relationship between delayed onset of SWC and worse MRI Barkovich score, these findings are not reliable and should be interpreted with great caution.



**Figure 88** Correlation between time to onset of sleep wake cycling (SWC) and MRI scoring systems in infants with neonatal encephalopathy. There is a significant positive correlation between longer time to onset of SWC and higher Barkovich score ( $p = 0.005$ ,  $r_s = 0.454$ ) (A), NICHD ( $p = 0.001$ ,  $r_s = 0.526$ ) (B), and de Vries scores ( $p = 0.001$ ,  $r_s = 0.536$ ) (C).

### Correlation of SQ with MRI scoring systems

There was a significant negative correlation between lower SQ score and higher Barkovich (Figure 89A), NICHD (Figure 89B), and de Vries scores (Figure 89C). These all indicated that lower SQ score is correlated with greater abnormalities on MRI brain in NE. However, there is very wide variation in MRI results, and that there is not a predictable relationship between these variables. Therefore, although these findings suggest that there may be an overall relationship between worse SQ and worse MRI Barkovich score, these findings are not reliable and should be interpreted with great caution.



**Figure 89** Correlation between time to onset of sleep quality (SQ) and MRI scoring systems in infants with neonatal encephalopathy. There was a significant negative correlation between lower SQ score and higher Barkovich ( $p = <0.001$ ,  $r_s = -0.642$ ) (A), NICHD ( $p = <0.001$ ,  $r_s = -0.688$ ) (B), and de Vries scores ( $p = <0.001$ ,  $r_s = -0.658$ ) (C).

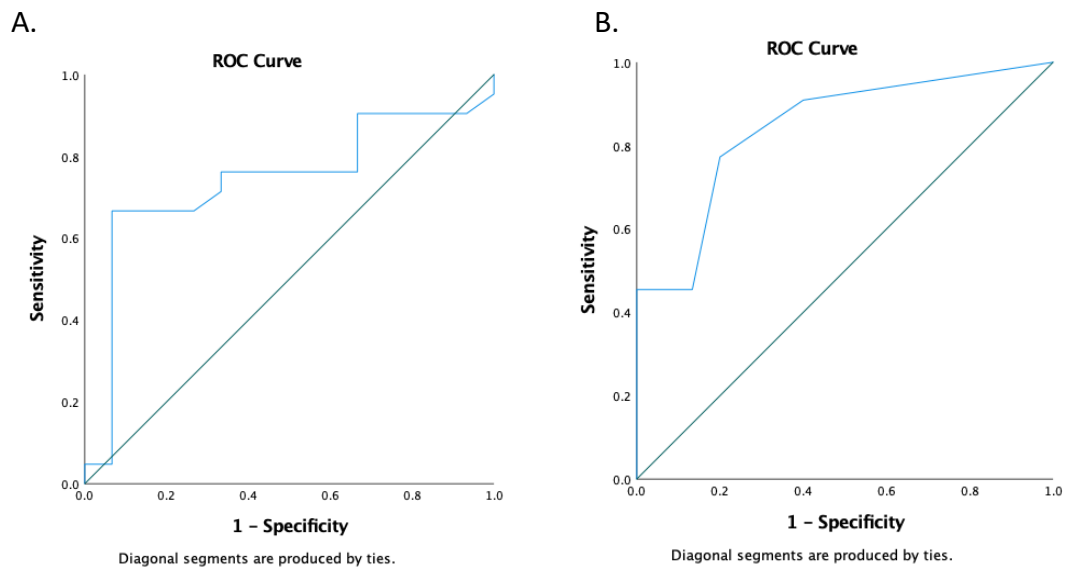


Comparison of discriminatory ability of time to SWC and SQ score to predict adverse outcome in neonatal encephalopathy

The calculated c-index for longer duration to SWC to predict adverse outcome in NE was 0.74 (95% CI 0.57 to 0.91) (Figure 90A). A time of 7.25 hours to SWC gave a sensitivity of 76% and a specificity of 67% to predict adverse outcome in NE. A time of 11 hours to SWC gave a sensitivity of 67% and a specificity of 80%.

The calculated c-index for higher SQ score to predict adverse outcome in NE was 0.84 (95% CI 0.71 to 0.97) (Figure 90B). A SQ score of 11.5 gave a sensitivity of 77% and a specificity of 80% to predict adverse outcome in NE. A SQ score of 12.5 gave a sensitivity of 91% and a specificity of 60%.

Compared to time to onset of sleep wake cycling (SWC), sleep quality (SQ) as measured by Burdjalov scoring system provided higher sensitivity (77% vs 67%), specificity (80% vs 73%) to predict adverse outcome in NE, and higher interobserver agreement (W = 0.92 vs W = 0.86) (Table 29).



**Figure 90 Predictive accuracy of time to sleep wake cycling (SWC) and sleep quality score (SQ) to predict adverse outcome in neonatal encephalopathy.** Receiver-operating-characteristic curves and the corresponding areas under the curve are shown for the measurement of time to SWC in hours (A) and SQ scores (B) and the sensitivity and specificity of this measure to predict adverse outcome. The calculated c-index for longer duration to SWC to predict adverse outcome in NE was 0.74 (95% CI 0.57 to 0.91,  $p = 0.015$ ) (A). The calculated c-index for higher SQ score to predict adverse outcome in NE was 0.84 (95% CI 0.71 to 0.97,  $p < 0.001$ ) (B).

	Time to SWC	SQ Score
Sensitivity	67%	77%
Specificity	73%	80%
Positive PV	78%	85%
Negative PV	61%	71%
Kendall's W	0.86 ( $p = 0.002$ )	0.92 ( $p < 0.001$ )

**Table 29 Comparison of the discriminatory ability of time to sleep wake cycling (SWC) and sleep quality (SQ) score to predict outcome in infants with neonatal encephalopathy.** Compared to time to onset of SWC, SQ score provided higher sensitivity, specificity, negative and positive predictive value for adverse outcome in patients with NE, and higher interobserver agreement.

#### Interobserver agreement of time to sleep wake cycling and sleep quality score

As previously demonstrated the collected data for the predictor variables was not normally-distributed. Therefore, the interobserver agreement between the assessors of time to SWC and SQ score was measured by the non-parametric test, Kendall's concordance coefficient ( $W$ ). The interobserver agreement for time to SWC demonstrated  $W = 0.86$ , and there was statistically significant agreement between observers. The interobserver agreement for SQ score demonstrated  $W = 0.92$ , and there was statistically significant agreement between observers. Therefore, both measurements demonstrated good levels of interobserver agreement, with slightly better agreement for SQ scores.

#### Association with longer duration to SWC and worse sleep quality with inflammatory cytokines

As disrupted sleep is associated with both recovery from inflammatory insults and worse outcome in NE, we examined the association between time to SWC and SQ score and inflammatory cytokines in infants with NE. As the predictor and outcome data was not normally distributed, the non-parametric Spearman's correlation was used to examine the time to SWC and SQ scores and cytokine levels.

No significant correlations were found between time to onset of SWC and cytokine levels in infants undergoing TH for NE ( $n = 37$ ; Table 30A). However, in infants with unfavourable outcomes ( $n = 21$ ) a longer duration to SWC was associated with higher EPO and IL-8 infants undergoing TH for NE (Table 30B).

Lower sleep quality score was correlated with higher EPO and IL-1 $\beta$  (n= 37; Table 31A). However, in infants with unfavourable outcome (n = 21) lower SQ score was associated with higher TNF- $\alpha$  in infants undergoing TH for NE (Table 31B).

A	Cytokine	Spearman (r)	p-value	B	Cytokine	Spearman (r)	p-value
	EPO	0.20	0.27		* EPO	0.63	0.03
	GMCSF	0.32	0.86		GMCSF	0.14	0.66
	IFN- $\gamma$	0.19	0.31		IFN- $\gamma$	-0.03	0.93
	IL-1 $\alpha$	0.12	0.58		IL-1 $\alpha$	0.17	0.67
	IL-1ra	0.12	0.95		IL-1ra	0.43	0.16
	IL-1 $\beta$	-0.01	0.94		IL-1 $\beta$	0.09	0.77
	IL-6	0.05	0.77		IL-6	0.42	0.13
	IL-8	0.05	0.74		* IL-8	0.56	0.03
	IL-10	0.00	0.99		IL-10	0.32	0.27
	IL-18	0.09	0.61		IL-18	0.28	0.38
	TNF- $\alpha$	0.12	0.51		TNF- $\alpha$	0.35	0.22
	TNF- $\beta$	0.08	0.71		TNF- $\beta$	0.66	0.08
	VEGF	0.17	0.35		VEGF	0.48	0.10

**Table 30** Correlation between time to sleep wake cycling (SWC) and cytokines in all infants with neonatal encephalopathy (A), and correlation between SWC and cytokines in infants with unfavourable outcome following neonatal encephalopathy (B), as measured by Spearman correlation coefficient. In infants with unfavourable outcomes a longer duration to SWC was positively associated with EPO and Interleukin (IL)-8.

\* indicates a significant correlation with the level of significance set at  $p < 0.05$ .

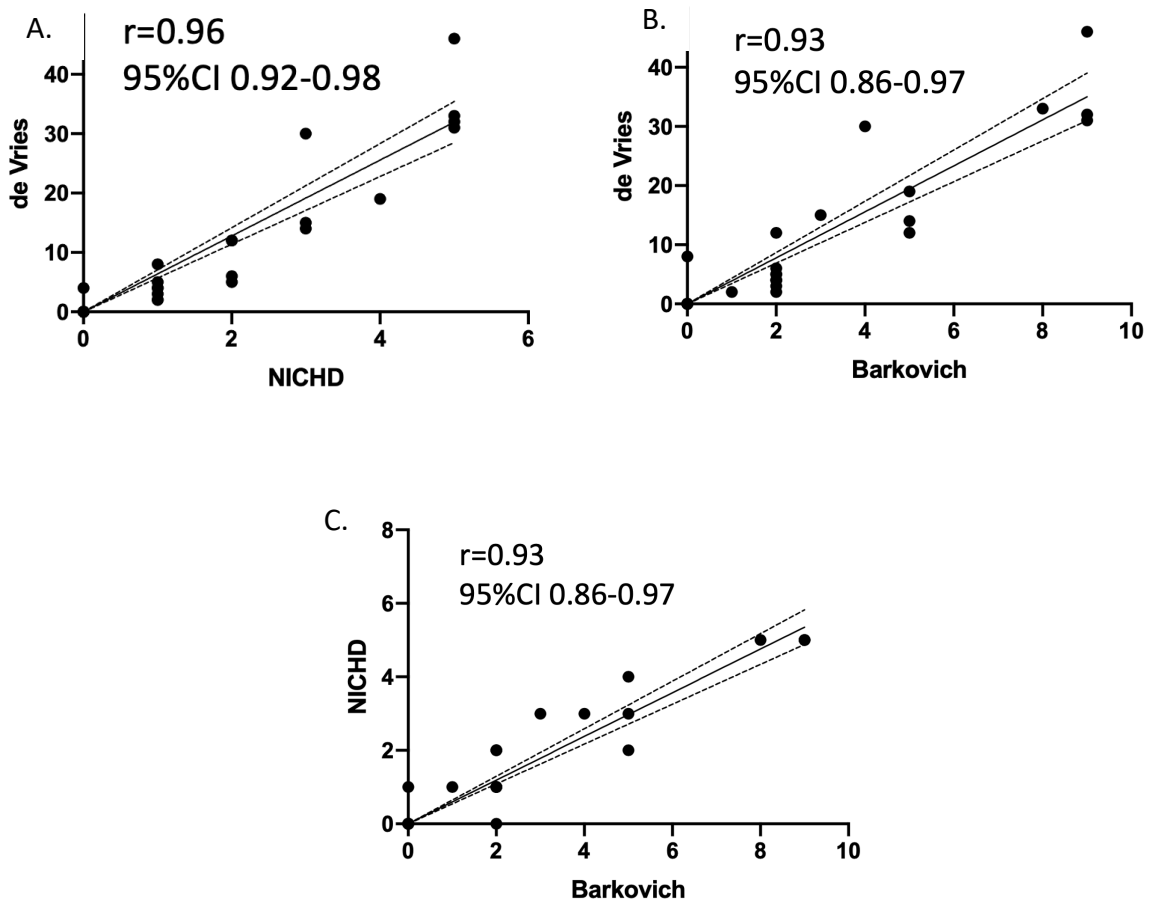
A	Cytokine	Spearman (r)	p-value	B	Cytokine	Spearman (r)	p-value
	* EPO	-0.42	0.02		EPO	-0.49	0.10
	GMCSF	-0.14	0.44		GMCSF	-0.20	0.52
	IFN- $\gamma$	-0.32	0.07		IFN- $\gamma$	-0.21	0.49
	IL-1 $\alpha$	-0.23	0.27		IL-1 $\alpha$	-0.41	0.28
	IL-1ra	-0.19	0.28		IL-1ra	-0.51	0.09
	* IL-1 $\beta$	-0.35	0.04		IL-1 $\beta$	-0.39	0.19
	IL-6	-0.14	0.91		IL-6	-0.34	0.24
	IL-8	-0.20	0.06		IL-8	-0.51	0.06
	IL-10	-0.25	0.15		IL-10	-0.53	0.05
	IL-18	-0.24	0.19		IL-18	-0.53	0.08
	TNF- $\alpha$	-0.25	0.16		* TNF- $\alpha$	-0.62	0.02
	TNF- $\beta$	0.19	0.34		TNF- $\beta$	-0.41	0.32
	VEGF	-0.08	0.68		VEGF	-0.51	0.07

**Table 31** Correlation between sleep quality score (SQ) and cytokines in all infants with neonatal encephalopathy (NE) (A), and correlation between SQ and cytokines in infants with unfavourable outcome following neonatal encephalopathy (B), as measured by Spearman correlation coefficient. In infants with NE undergoing therapeutic hypothermia (TH) higher SQ was negatively associated with EPO and interleukin (IL)-1 $\beta$ . In infants with unfavourable outcomes higher SQ was negatively associated with tumour necrosis factor (TNF)-  $\alpha$ .

\* indicates a significant correlation with the level of significance set at  $p < 0.05$ .

## Correlation of different MRI scoring systems

Different prognostic scoring systems are employed to assess abnormalities on MRI brain in infants with NE. We compared the results of MRI scoring systems for 35 infants with NE that were treated with TH. Full results for all 3 scoring systems were available for all infants. Correlations were examined using the non-parametric Spearman's correlation coefficient ( $r$ ). All scoring systems were closely correlated. NICHD and de Vries scoring systems showed the closest correlation,  $r = 0.96$  (95% CI 0.92 to 0.98) (Figure 91A), followed by both Barkovich and de Vries (Figure 91B) and Barkovich and NICHD scoring systems (Figure 91C), for both  $r = 0.93$  (95% CI 0.86 to 0.97).



**Figure 91** Correlation between MRI brain scoring systems in infants with neonatal encephalopathy; comparison of NICHD system and de Vries (A), Barkovich system and de Vries (B), and Barkovich system and NICHD (C).

## Discussion

We found that infants with NE and adverse outcome had longer duration to onset of sleep wake cycling (SWC) and worse sleep quality (SQ). Longer duration to SWC has been associated with adverse outcome in previously reported studies [92, 549]. The definition of time of onset to SWC by Takenouchi has previously been demonstrated to provide valuable prognostic information for infants with NE who are undergoing TH [549]. In a recent study reported the best overall prediction of outcome in NE with a combination of MRI and SWC on aEEG [552]. We found further evidence that time to SWC remains a good predictor of outcome in NE in the era of therapeutic hypothermia. We found a significant association between sleep quality (SQ), as measured by Burdjalov score [550], and outcome in NE. This SQ score has previously been demonstrated to be associated with improved cerebral maturity and SQ in preterm infants [550] but to our knowledge it had not previously been applied to infants with NE. SQ score provided greater sensitivity and specificity to predict adverse outcome in NE compared to time to SWC. The evidence presented suggests that SQ score provides improved discriminatory ability to predict adverse outcome in NE when compared to time to SWC. However, the very wide confidence intervals for the c-index for both time to SWC and SQ, and the irregularity of the ROC curves, including some overlap beyond the line of random chance, demonstrate that the sample size is inadequate to draw any definitive conclusions. The evidence is to date is suggestive, but it is presumptive to draw this definitive conclusion based on the evidence currently available, as the sample size is too small and the evidence is not sufficiently robust. To reliably answer this question requires the same methodology to be repeated with a larger sample size. There was greater



interobserver agreement for SQ score than time to SWC. However, the discriminatory ability of either SQ or SWC to predict outcome in NE was still inferior to MRI [211], but requires less resources and provides earlier prognostic information than MRI.

We found that better SQ was negatively associated with serum EPO and IL-1 $\beta$  levels, and that in patients with unfavourable outcomes shorter duration to onset of SWC and is associated with higher EPO levels and that higher SQ was associated with lower TNF- $\alpha$  levels. There is evidence of a bidirectional relationship between disrupted sleep and dysregulated inflammation [480], including alterations in cytokine production [553]. It remains unclear whether sleep disruption is a consequence of worse brain injury following NE or whether sleep may play a causal role in worsening dysregulated inflammation and outcome in NE. However, there is biological plausibility that disrupted sleep may play a causal role and this requires further investigation. Sleep disruption has been associated with increased EPO levels in other infant cohorts [554] and although EPO was proposed as a treatment for infants with NE, a recent large trial of EPO administration was associated with increased risk of adverse outcome in NE [141]. Both IL-1 $\beta$  and TNF- $\alpha$  promote sleep, particularly NREM sleep [555], and are associated with adverse outcome in NE [118]. Therefore, it is not surprising that an association was found in this study, and that their levels showed an inverse relationship to sleep quality. It remains unclear however, in which direction this relationship occurs. Several factors associated with sleep onset are disrupted during care in NICU, including excessive light exposure [94], excessive noise exposure [556], and disruption to feeding patterns [546]. These factors are modifiable. Although no study

has demonstrated any intervention to improve sleep in NICU [547], light-dark cycling has demonstrated improved outcomes in infants born prematurely [93]. With a sufficient sample size, it may be possible to statistically control for other early prognostic biomarkers and better isolate the association between sleep and outcomes in NE. Our intention was to control for the severity of NE for a more direct examination of the relationship between worse sleep states and dysregulated inflammation or adverse outcome. The purpose was to assess more specifically if adverse sleep may be a contributing factor to dysregulated inflammation and adverse outcome. The best measure to assess the severity of NE in the first few days is standardised clinical exam and Sarnat staging. Unfortunately, there were insufficient numbers of infants in each group to control for severity of NE. Questions remain whether longer duration to onset of SWC or worse sleep quality reflects worse brain injury or whether circumstances that interfere with sleep onset and quality may exacerbate dysregulated inflammation and lead to worse outcomes in NE. Sleep disruption persists in NE [557] and early interventions to improve it may provide improved long-term outcomes [90].

Two of the most commonly used scoring systems (Barkovich, NICHD) and one recently validated scoring system (de Vries) that includes more advanced imaging techniques were compared for their level of correlation. The purpose was to assess if there is a close correlation between the scoring systems so that studies that report different systems can be more accurately compared. We demonstrated close correlation with all 3 MRI scoring systems. They are similar in many ways and in the anatomical areas assessed,

however they vary significantly in complexity and time required for completion. Chapter 3 demonstrated that many different scoring systems are currently in use, which limits the ability to generalise conclusions from these studies and to synthesise results from different studies. These results demonstrate that the scores are comparable and so results can be interpreted as such too. These results were confirmed in a larger study that assessed their correlation to neurodevelopmental outcome and demonstrated high specificity for adverse outcome. However, they were unable to identify which system best predicted adverse outcome at 2 years [382].

## Chapter 9: Discussion

Neonatal encephalopathy (NE) is a devastating condition for many infants and their families. The condition remains incompletely understood [420] and currently available therapies are only partially effective [204] and unsuitable for many [5]. In this study we explored several aspects of the condition that may lead to improved definition and recognition of the condition, the development of new therapeutic options for NE, and for improved prognostication for those with NE.

### [Systematic review of terminology, definitions, and diagnostic criteria in trials for neonatal encephalopathy](#)

In Chapter 2 we analysed the terminology, definitions, and eligibility criteria employed in trials for NE. We found that hypoxic ischaemic encephalopathy (HIE) remained the most frequently used terminology to describe the condition, despite efforts to use the more broadly encompassing term NE [8]. Several papers used different terms interchangeably. Few studies used or referenced a definition of the condition and there was no consistency in the definition of the term used between trials. Eligibility criteria were consistent across several inclusion criteria, such as the use of acidosis on cord or early postnatal sampling and low Apgar scores, however there was significant variation in their implementation. We found significant variation in the application of the criteria for neurological dysfunction, which has major implications for those deemed eligible for interventions, even when these variations are very subtle [205].

Improved common understanding of the condition, terminology used, and diagnostic criteria would provide clarity for diagnostic purposes with medico-legal implications, improve generalisability of results from future RCTs, and allow greater comparison of care and outcomes between settings. This work is the first step towards a consensus building project and was presented at the first consensus steering group meeting. The purpose is to provide a background to inform the first stage of a Delphi technique to develop a consensus definition and diagnostic criteria. The Delphi technique is an established technique in the medical literature consensus building around a definition [7, 558, 559]. The technique involves a series of rounds in which a group of expert stakeholders initially provide their opinion the issue [560]. In future rounds the expert group receive all responses, including their own, to the initial round and give their opinion on the strength and weaknesses of the responses. The findings of each round are shared anonymously which minimises the effect of power-dynamics. With careful modulation, a final consensus meeting with an open-floor discussion to create a finalised definition is an established method of concluding the process. While a very large volume of work has been undertaken by an expert panel to form the ACOG/APP definition of NE [1], our study suggests that it has not gained significant traction to date. One benefit of the proposed process is the broader group of stakeholders that form part of the steering group and Delphi process - including clinical experts, research experts, and patient and family advocates – which may develop broader acceptance of the definition. The consensus project is being co-ordinated with an international group of experts from the Newborn Brain Society

(<https://newbornbrainsociety.org/>) and the James Lind Alliance

(<https://www.jla.nihr.ac.uk/>).

### Optimal MRI technique to predict long-term prognosis in NE: a systematic review and meta-analysis

In Chapter 3 we synthesised and examined the prognostic value of MRI and various MRI techniques to predict adverse outcome in NE. We found that MRI provides excellent prognostic value overall, but there is significant variation between techniques. Magnetic resonance spectroscopy (MRS) provided the optimum prognostic value compared to conventional imaging or diffusion weighted imaging. An additional benefit of MRS is that it does not depend on imaging being completed during a short window and provides accurate prognostic information over the first two weeks of life [380], unlike the other imaging techniques which have specific windows for optimal imaging which do not overlap. MRS also provides data on a dynamic process, which may provide guidance on treatment options in the future. However, the meta-analysis for MRS included the smallest number of studies and the wide confidence intervals. There was also very limited overlap of the regions imaged for MRS and the metabolites measured. These studies require external validation and studies of larger cohorts as the sample size, and specifically event rates, were very low in many studies.

MRI provides promise as a surrogate marker for later neurodevelopmental outcome in trials of interventions in NE, but further studies are crucial prior to implementation as a

trial endpoint [212]. Improved prognostic information is also crucial for improved parental communication, more accurate expectations in the postnatal period, and improved identification of those who most require further therapeutic interventions beyond the NICU. Pragmatic factors will likely influence the timing and technique of MRI available, but these should be standardised as far as practicable. Improved understanding of how timing and technique influence the prognostic value of MRI is critical. This can enhance further harmonization of MRI acquisition, reporting, and interpretation [551]. As this study has identified, amassing a sample size with sufficient power will be difficult without co-ordination of MRI technique and interpretation which is essential for improved understanding application of results to individuals with NE and their carers [561]. We extracted data from each study on postnatal timing of MRI. Aside from MRI technique, this factor has a major impact on the interpretation of MRI results. As discussed above, different MRI techniques have optimal windows for MRI acquisition. The next stage of this study is to analyse the impact of MRI timing on the prognostic value of MRI, and then to examine the influence of the combination technique and timing on prognostic value. We also extracted data on MRI scoring systems, and we intend to analyse their effects on prognostic value too. Although several MRI scoring systems with good prognostic value have been developed [382], there is currently no international guideline or standard for MRI protocol for infants with NE. Such a guideline would significantly improve parent and carer counselling for infants with NE and clinical research in NE. We hope this work will provide input when such a guideline is developed.

## Biomarkers to predict outcome in neonatal encephalopathy: a systematic review and meta-analyses

In recent years a huge volume of work and resources have been invested in identifying blood and cerebrospinal biomarkers for prognosis in NE and we examined the current evidence in Chapter 4. We identified many eligible studies for inclusion in a meta-analysis, however variations in reporting technique severely limited the number that could be included in a meta-analysis. We found that serum IL-6, neuron-specific enolase, and TNF- $\alpha$  were all lower in participants with normal outcome. However, there was significant variation between included studies for each meta-analysis. Nonetheless, these results provide some prognostic value, improved understanding of the disease pathophysiology, and identification of potential targets for therapeutic interventions.

The EQUATOR Network (<https://www.equator-network.org/>) provides a range of guidelines for conducting clinical research and identifies specific guidelines for the uniform reporting of prognostic biomarkers in future studies of neurological disorders [562]. If followed would allow greater transparency in biomarker research, greater inclusion of studies in meta-analyses of biomarkers, more certainty regarding meta-analysis results, and less research waste. If a greater number of studies and participants could be included in future meta-analyses of biomarkers in NE, the impact of other factors that influence biomarker levels could be examined. These include the impact of timing of postnatal sampling on biomarkers to better identify the phases of injury in NE, and how the pathophysiology varies between each stage [420]. Other factors to be examined include the presence of chorioamnionitis which has distinct pathophysiology compared to sterile



hypoxia-inflammation in NE and response to treatments such as TH [563]. If future studies are enhanced with harmonisation of biomarker acquisition and reporting, it would significantly improve our understanding of disease pathophysiology and provide more accurate and specific prognostic value for individual infants.

#### [Cochrane Review: Melatonin treatment for infants with neonatal encephalopathy](#)

We synthesised and examined the evidence for melatonin treatment in infants with NE as a monotherapy and as an adjunctive treatment to TH in Chapter 5. We identified 4 randomised controlled trials of melatonin in NE, however there was significant variation between studies in the methodologies employed. All studies reported improved outcomes with melatonin treatment. However, only one pilot study of melatonin as an adjunctive therapy to TH, compared to placebo and TH, reported the primary outcome of a composite of death and adverse neurodevelopmental outcome [564]. They found no difference although the study was not powered to detect an effect. However, they reported significantly better cognitive outcomes in those treated with melatonin compared to placebo. Given these results and the robust pre-clinical evidence of neuroprotection from melatonin in NE there is an urgent need for rigorous and adequately powered phase 3 RCTs of melatonin in NE [196, 199].

This urgency is increased by the recent study of one of the most promising neuroprotective agents in NE, erythropoietin, which demonstrated no difference in outcomes between groups but an increased risk of adverse events in those treated with

erythropoietin [141], leaving melatonin as one of the next most advanced treatment options in research development for NE. We identified one ongoing phase III trial of melatonin in NE which is currently recruiting (NCT03806816), and we are aware that planning for further trials are ongoing. Partly through collaboration with international researchers on this study, it is intended that patients with NE in Irish hospitals will be invited to participate in these future clinical trials.

### Effects of melatonin on serum cytokines in neonatal encephalopathy

We explored the effects of melatonin treatment on serum cytokine levels in infants with NE and healthy controls in Chapter 6. We demonstrated significant effects of melatonin treatment in both cohorts compared to both vehicle samples and endotoxin-stimulated samples. Overall melatonin induced a complex alteration in cytokine production in infants with NE, with a significant reduction in GM-CSF and IFN- $\gamma$  and a significant increase in IL-1RA, IL-8, TNF- $\alpha$ , and VEGF. Compared to the response to melatonin treatment in healthy controls, infants with NE were hyporesponsive to melatonin treatment for several inflammatory cytokines. We did not find significant diurnal variation in many cytokines, nor in the response to endotoxin-stimulation or melatonin treatment.

These results require greater scrutiny to assess the impact of time of postnatal sampling, the presence of chorioamnionitis, and differences in treatment response by sex for improved understanding of when melatonin treatment could be optimised and who is likely to benefit most from melatonin treatment. As melatonin is a highly lipophilic agent,

ethanol has traditionally been used as the excipient. However, ethanol itself may provide neuroprotection following hypoxia-ischaemia [565, 566]. In these experiments we used ethanol in our control, untreated samples to control for this effect. We will examine the difference in response to ethanol treatment compared to controls without ethanol in infants undergoing TH, recruited to a previous study [450]. Melatonin induced a complex cytokine response, with increases in both pro- and anti-inflammatory cytokines and it is difficult to establish a specific pattern of response. We will examine the effect of altered cytokine response on other functions of the innate immune system, such as inflammasome activation and ROS production. A large sample size of healthy controls and infants with NE should be examined to better elucidate any influence of circadian factors on diurnal variation in serum cytokines.

#### Effects of melatonin treatment on the innate immune system in neonatal encephalopathy

In Chapter 7 we explored the effects of melatonin on several aspects of the innate immune system in infants with NE. We examined the effect of melatonin treatment on neutrophil and monocyte phenotype, mRNA gene expression inflammasome related genes and circadian rhythm genes, and on the expression of microRNA. Despite evidence that melatonin is a potent immune-modulating agent in other populations, we did not find this to be the case in infants with NE. Melatonin reduced TLR-2 expression in endotoxin-stimulated samples, and increased gene expression of NLRP3 inflammasome with no associated rise in inflammasome-related cytokines. Otherwise, we found no difference in

neutrophil or monocyte phenotype, nor any difference in inflammasome, circadian, or mircoRNA expression. This lack of significant differences in the innate immune system in NE may be due to the use of whole blood samples rather than single cell analysis, variable timing of sampling over the first week of life, and a small sample size for some experiments. A further limitation is a lack of comparison of the effects of melatonin treatment in healthy controls. Although we did not find many significant changes with NE, melatonin appears to offer neuroprotection in NE (Chapter 5).

Further exploration of this mechanism of action is required and should include examination of the NLRP3 inflammasome and the effects of melatonin in the very early postnatal period, preferably within the first 24 hours of life if feasible. These responses require comparison with healthy controls. While pre-clinical studies have demonstrated that optimal neuroprotection from melatonin requires administration shortly after the hypoxic-ischaemic insult, no identified studies administered melatonin after 2 hours so the therapeutic window for melatonin treatment remains unclear [567]. Several large-animal models of NE have examined the neuroprotective effects of melatonin treatment [196, 568]. To date the best identified mechanism of action of melatonin in animal models is its anti-oxidant effects [569]. Melatonin acts as an anti-oxidant in receptor-dependent and -independent mechanisms and both of these pathways require further exploration to elucidate which mechanism is the most effective. Other mechanisms of action from pre-clinical models should be correlated with the findings from this study. The effects of melatonin treatment on the production of ROS by neutrophils also requires exploration. The

effect of ROS production on neutrophil function, in particular the form of cell death NETosis, has not been explored in NE. NETosis has been implicated in worsening injury following neonatal sepsis [570]. Modulation of this process by melatonin also requires exploration. As above, ethanol was included in the untreated vehicle samples for these experiments. A previous cohort of infants with NE undergoing TH were recruited and their innate immune response was examined without exposure to ethanol [43]. We will examine the differences in samples treated with and without ethanol. Several RCTs of melatonin treatment for infants with NE have been proposed and analysing the in vivo effects of melatonin treatment on the innate immune system in these participants would provide valuable evidence regarding the mechanism of action and should be included in the study design.

### [Sleep, inflammation, and outcomes in neonatal encephalopathy](#)

The significance of sleep on immune function and health outcomes has become increasingly evident in recent years and we explored this effect in infants with NE in Chapter 8. We found that patients with NE and adverse outcome had longer duration to onset of sleep-wake cycling and worse sleep quality than those with good outcomes. It remains unclear whether there is a causal relationship for this association.

There is strong plausibility that dysregulated sleep may impair the innate immune response and lead to adverse outcomes [480], and many aspects that entrain the sleep and the circadian rhythm are disrupted in the NICU for infants with NE [90]. While no intervention has been demonstrated to improve sleep in NICU [547], several circadian

rhythm entrainment factors are amenable to modification and have demonstrated improved outcomes in other neonatal populations. More broadly, sleep, the circadian rhythm and the factors which entrain it in the neonatal period are very poorly understood [85]. Future studies to examine the effect of light-dark cycling, the effect of feeding patterns, the effect of breastfeeding or exposure to human milk, and human milk constituents such as hormones that entrain the circadian rhythm require evaluation to optimise care in the NICU and beyond. Light-dark cycling improves outcomes for preterm infants [93] and is recommended by several neonatal societies [95]. This would be a worthwhile trial for newborns with NE. Exposure to enteral feeding, another major entrainment factor, varies significantly between clinical sites for infants undergoing TH, and many infants do not receive enteral feeds during TH [571]. However, evidence suggests that this is safe and leads to improved outcomes, including higher survival to discharge [546]. Enteral feeding may also expose infants to melatonin from breastmilk, and circadian fluctuations in this hormone and others involved in CR entrainment [572]. The effect of feeding practices on sleep and CR entrainment in neonates is poorly understood, but better understanding could lead to improved outcomes for a range of neonatal conditions including patients with NE and preterm infants. Correlation of sleep quality and time to SWC should be studied in those with and without enteral feeding during TH, and for those exposed to breastmilk or artificial formula to assess for any differences between groups.

## Conclusion

Neonatal encephalopathy remains poorly defined and incompletely understood. Although early identification of those with NE at risk of adverse outcome is essential, prognostication remains a significant challenge. MRI and MRS provide excellent prognostic value but require further evaluation to optimise their value. There is an urgent need for adjunctive therapies to therapeutic hypothermia in high-resource settings and other treatment options in low- middle- income countries, and melatonin is a promising therapy in these settings.

# Appendix

## I. Ethical approval



Prof Eleanor Molloy  
Consultant Neonatologist  
OLCHC and Coombe Women and Infants University Hospital  
*Eleanor.Molloy@tcd.ie*

25th May 2019

**Re: Study No. 12 – 2016 – Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group) – Amendments (amendments letter dated 28/2 2019)**

Dear Prof Molloy,  
Amendments to your study '**Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)**' were discussed by the Research Ethics Committee (REC). It was consensus of the REC that in the case of clinical suspicion for seizure activity, the aEEG recording should be unblinded. There is a typo in the paragraph 'At the End of the Study' of PIL. It states at the moment that there will be a change regarding treatment despite this being monitoring study. This should be amended accordingly. The amendments of your study were approved by the REC.

Yours sincerely

A handwritten signature in black ink, appearing to read 'J Miletin', is written over a light blue horizontal line.

Professor Jan Miletin (IMC 241348)  
Consultant Neonatologist  
Chairman of the Research Ethics Committee  
Coombe Women and Infants University Hospital (*jmiletin@coombe.ie*)





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Máistir/ Master: Dr. Rhona Mahony

**PRIVATE AND CONFIDENTIAL**

Professor Eleanor Molloy,  
Neonatology Consultant &  
Professor of Paediatrics,  
Trinity College,  
Dublin 2.

27<sup>th</sup> June 2016.

**Re: Neonatal Inflammation and Multiorgan dysfunction and Brian injury research group.  
(EC 14.2016)**

Dear Eleanor,

I am happy to inform you that the above study has now received ethical approval.

Kind regards,  
Yours sincerely,

  
Dr. John Murphy  
Chairman,  
Ethics Research Committee



29<sup>th</sup> June, 2017.

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Prof. Eleanor Molloy  
Dept. of Paediatrics, Trinity College Dublin,  
Trinity Centre for Health Sciences,  
National Children's Hospital, AMNCH,  
Tallaght,  
Dublin 24.

**Our ref:** REC-2016-028 *(please quote this reference on all correspondence)*  
**Re:** Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)

Dear Eleanor,

Many thanks for the amended documentation received in relation to the above research. I am pleased to advise that the requirements set out by the Committee in respect of your study have now been met. This being the case, ethical approval for the research is granted and it may now commence.

You are requested to submit a progress report to the Committee in twelve months, and annually thereafter as applicable. We would also like to know when and where you publish or present your results. Please be aware of your responsibilities with respect to the Hospital's good research practice policies and guidelines.

Kind regards.

Yours sincerely,

Dr. Maeve Eogan,  
Acting Chairman,  
Research Ethics Committee.



## II. Patient information leaflets

### Patients with NE



Consent: NICU

**Title of Study: Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)**

**Short Title: Inflammation and the Newborn Brain**

#### **Investigators:**

Principal Investigator: Professor Eleanor Molloy of Trinity College Dublin

Co-investigators: Prof Martin White, Prof John O'Learys, Dr Jan Miletin.

#### **Data Controllers:**

Data Controller's/joint Controller's Identity: Trinity College Dublin

Data Controller's/joint Controller's Contact Details: dataprotection@tcd.ie

Data Protection Officer's Identity: Mr. John Eustace

Data Protection Officer's Contact Details: dataprotection@tcd.ie

Your baby is being invited to take part in a study as he/she is admitted to the neonatal intensive care unit. However, before you decide whether or not to take part, it is important that you fully understand what the research is about and what you will be asked to do. It is important that you read the following information in order to make an informed decision and if you have any questions about any aspects of the study that are not clear to you, do not hesitate to ask me. Please make sure that you are satisfied before you decide to take part or not. Thank you for your time and consideration of this invitation

#### **Purpose of the Research Study**

This study is about finding out more about the brain health and wellbeing of babies admitted to the unit under our care. We are studying big babies who are off form and not feeding well after birth along with well babies who have not required any help after birth.

### **Why as a Participant/Respondent have I been asked to take part in this study?**

You have been asked to include your child in this study as he/she has been admitted to the neonatal intensive Care Unit. This study will be done in the intensive care unit at the Rotunda Hospital, the Coombe Womens and Infants University Hospital and the National Maternity Hospital.

### **The voluntary nature of participation:**

Taking part in this research study is entirely up to you and if you do decide for your baby to take part you will be provided with an information leaflet to take with you. Additionally, you will be required to sign a consent form. However, if you do not wish to take part and if you change your mind at any time (prior to publication), you can withdraw your baby from the Research Study without giving a reason.

### **During the Study**

The study essentially involves all aspects of routine care. We would be grateful if you would allow the study group to use the data from some of the following tests if they are required aspart of routine care for your baby:

1. Examining the baby's brain waves over the first 72 hours of life using a routine technique called EEG (Electroencephalogram) whereby electrical brain wave activity can be measured from your baby and the tracing is recorded on a machine. This test is painless and will not cause any discomfort to your baby. It is part of routine care for many babies in the Neonatal Intensive Care Unit.
2. Your baby may have an MRI brain scan if required as part of their routine care and this does not involve radiation.
3. We also make sure we have checked all the parts of the body such as the kidneys, lungs and heart using ultrasound techniques such as echocardiography.
4. The other case information and tests that are routinely performed on the babies in the unit (e.g urine sample collection) and the placenta will also be assessed. For example, if a spinal fluid sample is taken as part of routine care, a small amount of this sample will be set aside for the study.
5. We will follow your baby as they grow and mature to see how their development is progressing.

In addition we would ask to examine saliva and blood samples at a few different times. The first sample is from blood taken from the placenta/afterbirth. This would normally be discarded after birth. Baby blood samples would not be additional samples as a portion of

the blood sample taken as part of the baby's routine care will be set aside for the study, using international guidelines to ensure this does not affect your baby.

Saliva is not usually tested in babies. We would collect it by placing a swab into your baby's mouth until the swab is wet. It a safe procedure and it is not uncomfortable or painful for your baby.

Maternal sample: In order to understand the effects on inflammation on both mother and baby we request a sample of blood from you/your partner.

If having considered the study you decide not to participate, we assure you that in no way will your baby's care be affected. You may also decide to participate in some parts of the study and not all.

### **Potential harms/risks**

There are no potential/harms or risks to your baby from these tests which are in routine practice. Blood samples will only be taken with other bloods if needed as a routine part of your baby's day-to-day care and this will be decided by the consultant on-call who is caring for your baby.

### **Potential Benefits**

**To individual babies:** The benefit to your baby would be to assess the way the organs in the body function. Although many of the tests are part of routine care this study involves more detailed analysis of the results. We also measure inflammation as a way of predicting outcome.

**To Society:** Having a better understanding of inflammation and individual organ function will improve the treatments and tests available. New therapies to target inflammation could reduce complications for babies admitted to the NICU. By understanding inflammation in these babies we can target new treatments to add to cooling therapy to protect their brain and improve outcome.

### **Data Protection**

We will be using you and your baby's personal information in our research to help us understand newborn brain health. We will respect you and your infant's privacy. We will process this data in compliance with General Data Protection Regulation 2016 and the Health Research Regulations 2018. No information about who your infant is will be given to anyone or be published. The data produced from this study will be stored in a secure, locked location. Once the information is coded and no longer identifiable some data may be shared with collaborating researchers in University College Cork. Only members of the research team will have access to the data. You have a right to request access to you and you baby's data and a copy of it at any point during the study and have a right to have any inaccurate information corrected or deleted, and you can request to move your data from

one controller to another. You can decide to withdraw your consent for this study at any time, and all relevant data will be destroyed. You have a right to lodge a complaint with the Data Protection Commissioner regarding the study if you are unhappy. Following completion of the research study the data will be kept as long as required by the Hospital policy. The data will then be destroyed according to this same policy.

#### **Consent for Future Contact**

If you decide for your baby to take part in some or all of the study, we would request your permission to contact you again in the future. We hope to offer you and your baby specialised assessment of your baby's development at around 2 years of age. This is additional testing that is not part of the usual care for babies in NICU. If you are happy for us to keep your contact details for this part of the study, you are under no obligation to take part if contacted at a later date.

#### **At the End of the Study**

As this is just a monitoring study, there will be no change regarding treatment, which continues as necessary. We will retain the information obtained from this study (which is anonymised) for a period of 5 years. We will also use the summarised data of all the babies enrolled to plan a future study. Only summarised data of all the infants grouped together will be used for the purpose of future planning. We will not use individual baby data for any future planning or publications. We plan to publish the results of the study in a medical journal.

#### **Other considerations**

The study team may require access to your medical records and those of your baby. Again, any information collected will be completely anonymised.

#### **Contact Details**

If you have any questions about the study, call Prof Eleanor Molloy, consultant neonatologist, through the hospital Switch board.

## Healthy controls



**Title of Study:** Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)

**Short Title:** Inflammation and the Newborn Brain

**Investigators:**

Principal Investigator: Professor Eleanor Molloy:

Co-investigators: Prof Martin White, Prof John O'Leary, Dr Jan Miletin.

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Data Protection Officer's Identity: Mr. John Eustace

Data Protection Officer's Contact Details: dataprotection@tcd.ie

Your baby is being invited to take part in a study as he/she is a normal healthy infant. However, before you decide whether or not to take part, it is important that you fully understand what the research is about and what you will be asked to do. It is important that you read the following information in order to make an informed decision and if you have any questions about any aspects of the study that are not clear to you, do not hesitate to ask me. Please make sure that you are satisfied before you decide to take part or not. Thank you for your time and consideration of this invitation

**Purpose of the Research Study**

This study is about finding out more about the brain health and wellbeing of babies admitted to the unit under our care. We are studying big babies who are off form and not feeding well after birth along with well babies who have not required any help after birth.

**Why as a Participant/Respondent have I been asked to take part in this study?**

You have been asked to include your child in this study as he/she is a normal healthy baby. This study will be done at the Rotunda Hospital, the Coombe Womens and Infants University Hospital and the National Maternity hospital.

### **The voluntary nature of participation:**

Taking part in this research study is entirely up to you and if you do decide for your baby to take part you will be provided with an information leaflet to take with you. Additionally, you will be required to sign a consent form. However, if you do not wish to take part and if you change your mind at any time (prior to publication), you can withdraw your baby from the Research Study without giving a reason.

### **During the Study**

The study essentially involves all aspects of routine care. We would be grateful if you would allow the study group to use the data from some of the following tests if they are required as part of routine care for your baby:

If your baby has a blood sample as part of their routine care, for example for mild jaundice, we would be grateful if you would consider giving part of the sample to this study. This would allow us to compare your healthy baby's inflammatory responses with babies who are unwell in the Neonatal intensive care unit.

In addition we would ask to examine saliva samples at a few different times. Saliva is not usually tested in babies. We would collect it by placing a swab into your baby's mouth until the swab is wet. It is a safe procedure and it is not uncomfortable or painful for your baby.

If having considered the study you decide not to participate, we assure you that in no way will your baby's care be affected. You may also decide to participate in some parts of the study and not all.

### **Potential harms/risks**

There are no potential/harms or risks to your baby from these tests which are in routine practice. Blood samples will only be taken with other bloods if needed as a routine part of your baby's day-to-day care and this will be decided by the consultant on-call who is caring for your baby.

### **Potential Benefits**

**To individual babies:** The benefit to your baby would be to assess the way the organs in the body function. Although many of the tests are part of routine care this study involves more detailed analysis of the results. We also measure inflammation as a way of predicting outcome.

**To Society:** Having a better understanding of inflammation and individual organ function will improve the treatments and tests available. New therapies to target inflammation could reduce complications for babies admitted to the NICU. By understanding inflammation in



these babies we can target new treatments to add to cooling therapy to protect their brain and improve outcome.

### **Data Protection**

We will be using you and your baby's personal information in our research to help us understand newborn brain health. We will respect you and your infant's privacy. We will process this data in compliance with General Data Protection Regulation 2016 and the Health Research Regulations 2018. No information about who your infant is will be given to anyone or be published. The data produced from this study will be stored in a secure, locked location. Once the information is coded and no longer identifiable some data may be shared with collaborating researchers in University College Cork. Only members of the research team will have access to the data. You have a right to request access to you and your baby's data and a copy of it at any point during the study and have a right to have any inaccurate information corrected or deleted, and you can request to move your data from one controller to another. You can decide to withdraw your consent for this study at any time, and all relevant data will be destroyed. You have a right to lodge a complaint with the Data Protection Commissioner regarding the study if you are unhappy. Following completion of the research study the data will be kept as long as required by the Hospital policy. The data will then be destroyed according to this same policy.

### **Consent for Future Contact**

If you decide for your baby to take part in some or all of the study, we would request your permission to contact you again in the future. We hope to offer you and your baby specialised assessment of your baby's development at around 2 years of age. This is additional testing that is not part of the usual care for babies. If you are happy for us to keep your contact details for this part of the study, you are under no obligation to take part if contacted at a later date.

### **At the End of the Study**

As this is just a monitoring study, there will be change regarding treatment, which continues as necessary. We will retain the information obtained from this study (which is anonymised) for a period of 5 years. We will also use the summarised data of all the babies enrolled to plan a future study. Only summarised data of all the infants grouped together will be used for the purpose of future planning. We will not use individual baby data for any future planning or publications. We plan to publish the results of the study in a medical journal.

### **Other considerations**

The study team may require access to your medical records and those of your baby. Again, any information collected will be completely anonymised.

**Contact Details**

If you have any questions about the study, call Prof Eleanor Molloy, consultant neonatologist, through the hospital Switch board.

### III. Parent or guardian consent form



**Date:** \_\_\_\_\_ **Subject Number:** \_\_\_\_\_

- I consent for my baby to take part in the above study.
- I confirm that I have read and understood the patient information leaflet.
- I am happy that my questions have been answered regarding the study.
- I understand that I may freely withdraw from the study at any time without my care or the care of my baby being affected.
- I explicitly consent to having my medical notes and my baby's medical notes examined as part of this study.
- I consent to having extra blood samples saved if/when routine blood sampling is being performed.
- I consent to having saliva samples collected from my baby.
- I consent to having urine samples collected from my baby.
- I consent for my placenta/afterbirth to be examined.
- I consent to having an EEG (Electroencephalogram) performed on my baby.
- I understand that I can withdraw my biological material at any time without any negative repercussions.
- I understand that my biological material will be disposed of in a lawful and respectful way.
- I consent to be re-contacted by researchers about possible future research related to the current study for which I may be eligible.
- I consent to take part in this research study having been fully informed of the risks, benefits and alternatives.

\_\_\_\_\_  
Name of Parent

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Parent

\_\_\_\_\_  
Name of Doctor

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Doctor

ACOG/AAP criteria to determine whether NE is due to peripartum or intrapartum hypoxia ischemia<sup>a</sup>.

---

1. Does the baby meet the definition for NE?

35+ weeks gestation, abnormal consciousness±difficulty initiating and maintaining respiration, seizures, abnormal tone/reflexes

2. What is the likelihood that an acute peripartum or intrapartum event was the major contributor?

Neonatal signs

a. Apgar score of less than 5 at 10 min of life

b. Fetal umbilical artery acidemia: pH < 7 or base deficit ≥12

c. MRI obtained between 24 and 96 h and up to day 10 showing distinct basal-ganglia-thalamus, watershed or near-total cortical injury pattern

d. Presence of multisystem organ failure – can include cardiac, renal, hepatic, metabolic, hematologic and gastrointestinal dysfunction

Type and timing of contributing factors consistent with an acute or peripartum event

a. Sentinel hypoxic or ischemic event immediately before or during labor/delivery: e.g., ruptured uterus, umbilical cord prolapse

b. Fetal heart rate pattern that deteriorates to absent variability with: recurrent late or variable decelerations, or with bradycardia, or a sinusoidal pattern for ≥20 min

c. MRI obtained between 24 and 96 h and up to day 10 showing distinct basal-ganglia-thalamus or watershed pattern

d. No evidence of other proximal or distal factors that could contribute substantially or indicate other underlying pathobiology e.g. abnormal fetal growth, congenital microcephaly, maternal infection, neonatal sepsis

Developmental outcome is spastic quadriplegic or dyskinetic cerebral palsy (CP)

a. Other CP subtypes are less likely to be associated with an acute peripartum or intrapartum event and, spastic quadriplegia and dyskinesia can also have other causes.

b. Other developmental disabilities may occur, but are not specific to acute peripartum or intrapartum events and may arise from a variety of causes

**Table 32 ACOG/AAP 2014 criteria for diagnosis of peripartum or intrapartum hypoxia ischaemia.** From *Neonatal encephalopathy: Focus on epidemiology and underexplored aspects of etiology*, Seminars in Fetal and Neonatal Medicine, 2021, S. McIntyre et al [12].

## V. Chapter 3 Appendices

### Search Strategy

**Table 33 Search strategy and results of the original and updated literature search**

#### Pubmed

1. Neonatal encephalopathy [1] OR Hypoxic Ischaemic Encephalopathy [1]OR Perinatal asphyxia [1]
2. Neonatal encephalopathy [tiab] OR Hypoxic Ischaemic Encephalopathy [tiab]OR Perinatal asphyxia [tiab]
3. #1 OR #2
4. MRI [1] OR Magnetic Resonance Imaging [1] OR MRS [1] OR Magnetic Resonance Spectroscopy [1]
5. MRI [tiab] OR Magnetic Resonance Imaging [tiab] OR MRS [tiab] OR Magnetic Resonance Spectroscopy [tiab]
6. #4 OR #5
7. Prognosis [1] OR Outcome [1] OR Result [1]
8. Prognosis [tiab] OR Outcome [tiab] OR Result [tiab]
9. #7 OR #8
10. #3 AND #6 AND #9

#### Embase

1. 'Neonatal encephalopathy'/exp OR 'hypoxic ischaemic encephalopathy'/exp OR 'perinatal asphyxia'/exp
2. ('Neonatal encephalopathy' OR 'hypoxic ischaemic encephalopathy' OR 'perinatal asphyxia'): ti, ab, kw
3. #1 OR #2
4. 'MRI'/exp OR 'magnetic resonance imaging'/exp OR 'MRS'/exp OR 'magnetic resonance spectroscopy'/exp
5. ('MRI' OR 'Magnetic Resonance Imaging' OR 'MRS' OR 'magnetic resonance spectroscopy'): ti, ab, kw
6. #4 OR #5
7. 'Prognosis'/exp OR 'outcome'/exp OR 'result'/exp
8. ('Prognosis' OR 'outcome' OR 'result'): ti, ab, kw
9. #7 OR #8
10. #3 AND #6 AND #9

#### Web of Science

1. TS=('neonatal encephalopathy' OR 'hypoxic ischemic encephalopathy' OR 'perinatal asphyxia')
2. TS=('MRI' OR 'magnetic resonance imaging' OR 'MRS' OR 'magnetic resonance spectroscopy')
3. TS=('prognosis' OR 'outcome' OR 'result')
4. #1 AND #2 AND #3

#### Cochrane library

1. [mh "neonatal encephalopathy"] OR [mh "hypoxic ischemic encephalopathy"] OR [mh "perinatal asphyxia"]
2. ("neonatal encephalopathy" OR "hypoxic ischemic encephalopathy" OR "perinatal asphyxia"):ti,ab,kw
3. #1 OR #2
4. [mh "MRI"] OR [mh "magnetic resonance imaging"] OR [mh "MRS"] OR [mh "magnetic resonance spectroscopy"]
5. ("MRI" OR "magnetic resonance imaging" OR "MRS" OR "magnetic resonance spectroscopy"):ti,ab,kw
6. #4 OR #5
7. [mh "prognosis"] OR [mh "outcome"] OR [mh "result"]
8. ('prognosis' OR 'outcome' OR 'result'):ti,ab,kw
9. #7 OR #8
10. #3 and #6 and #9

## MRI to predict outcome in NE, comparison of techniques

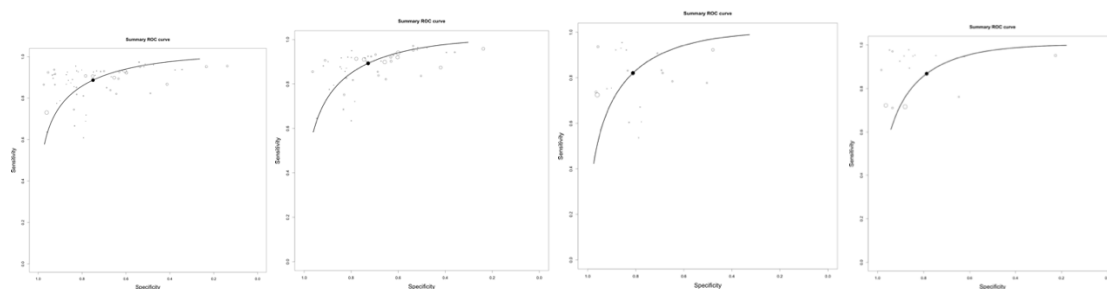


Figure 92 HSROC curve of total MRI, T1/T2, DWI, and MRS studies to predict long-term outcome in neonatal encephalopathy.

## VI. Chapter 4 Appendices

### Table 34 Search strategy and results of the original and updated literature search

#### EMBASE

1. 'biological marker'/exp OR 'cytokine'/exp (1,442,990) **1,399,203**
2. (Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*):ti,ab (621,309) **621599**
3. #1 OR #2 (1,575,348) **1,563,635**
4. 'hypoxic ischemic encephalopathy'/exp (4578) **4583**
5. (encephalopathy OR 'Ischemia-Hypoxia' OR 'perinatal asphyxia' OR 'fetal anoxia'):ti,ab (54999) **55018**
6. #4 OR #5 (57233) **57255**
7. 'newborn'/exp (524748) **524836**
8. (Neonat\* OR newborn OR infant\* OR perinatal):ti,ab (738,245) **738400**
9. #7 OR #8 (981,285) **981472**
10. #3 AND #6 AND #9 (998 hits) **995**

#### Update search 13.3.21

1. 'biological marker'/exp OR 'cytokine'/exp (1,442,990) **1,944,527**
2. (Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*):ti,ab (621,309) **952,657**
3. #1 OR #2 (1,575,348) **2,212,021**
4. 'hypoxic ischemic encephalopathy'/exp (4578) **8228**
5. (encephalopathy OR 'Ischemia-Hypoxia' OR 'perinatal asphyxia' OR 'fetal anoxia'):ti,ab (54999) **74,314**

6. #4 OR #5 (57233) **78,344**
7. 'newborn'/exp (524748) **614,864**
8. (Neonat\* OR newborn OR infant\* OR perinatal):ti,ab (738,245) **907,543**
9. #7 OR #8 (981,285) **1,179,503**
10. #3 AND #6 AND #9 (998 hits) **1,598**
11. #10 AND (2017:py OR 2018:py OR 2019:py OR 2020:py OR 2021:py) **621**

## PubMed

Original search 25.03.2017

1. "Biomarkers"[Mesh] OR "Cytokines"[Mesh] (1.240.612 hits) **1240790**
2. Biomarker\*[tiab] OR biological indicator\*[tiab] OR biological marker\*[tiab] OR serum marker\*[tiab] OR Immunologic Marker\*[tiab] OR Cytokine\*[tiab] (462470 hits) **462667**
3. #1 OR #2 (1424318 its) **1424629**
4. "Hypoxia-Ischemia, Brain"[Mesh] (4521hits ) **4522**
5. Encephalopathy[tiab] OR Ischemia-Hypoxia[tiab] OR perinatal asphyxia[tiab] OR 'fetal anoxia'[tiab] (39345) **39252**
6. #4 OR #5 (42256) **42164**
7. "Infant, Newborn"[Mesh] (545040) **545088**
8. Neonat\*[tiab] OR newborn[tiab] OR infant\*[tiab] OR perinatal[tiab] (637191) **637285**
9. #7 OR #8 (907620) **907734**
10. #3 AND #6 AND #9 (834 hits) **834**

Update search 13.03.2021

1. "Biomarkers"[Mesh] OR "Cytokines"[Mesh] (1.240.612 hits) **1,403,275**
2. Biomarker\*[tiab] OR biological indicator\*[tiab] OR biological marker\*[tiab] OR serum marker\*[tiab] OR Immunologic Marker\*[tiab] OR Cytokine\*[tiab] (462470 hits) **687,696**
3. #1 OR #2 (1424318 its) **1,711,111**
4. "Hypoxia-Ischemia, Brain"[Mesh] (4521hits ) **5,896**
5. Encephalopathy[tiab] OR Ischemia-Hypoxia[tiab] OR perinatal asphyxia[tiab] OR fetal anoxia[tiab] (39345) **50,431**
6. #4 OR #5 (42256) **53,899**
7. "Infant, Newborn"[Mesh] (545040) **619,625**
8. Neonat\*[tiab] OR newborn[tiab] OR infant\*[tiab] OR perinatal[tiab] (637191) **759,079**
9. #7 OR #8 (907620) **1,060,483**
10. #3 AND #6 AND #9 (834 hits) **1,209**

11. #3 AND #6 AND #9 2017-2021 403

## World of science

Original search 25.03.2017

1. TS=(Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*) (660,366) 664979
2. TS=(encephalopathy OR 'Ischemia-Hypoxia' OR 'perinatal asphyxia' OR 'fetal anoxia') (50,581) 66,057
3. TS=(Neonat\* OR newborn OR infant\* OR perinatal) (632,446) 631974
4. #1 OR #2 #3 (518 hits) 519

Update search 13.03.2021

1. TS=(Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*) (660,366) 991,395
2. TS=(encephalopathy OR 'Ischemia-Hypoxia' OR 'perinatal asphyxia' OR 'fetal anoxia') (50,581) 50,991
3. TS=(Neonat\* OR newborn OR infant\* OR perinatal) (632,446) 788,152
4. #1 AND #2 AND #3 (518 hits) 861
5. #1 AND #2 AND #3 2017 – 2021 349

## Cochrane Library

Original search 25.03.2017

1. [mh "Biomarkers"] OR [mh "Cytokines"] (33581) 33581
2. (Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*):ti,ab,kw (23391) 34905
3. #1 OR #2 (48313) 50830
4. [mh "Hypoxia-Ischemia, Brain"] (149) 149
5. (encephalopathy or 'Ischemia-Hypoxia' or 'Ischemia Hypoxia' or 'perinatal asphyxia' or 'fetal anoxia'):ti,ab,kw (2405) 2405
6. #4 OR #5 (2405) 2405
7. [mh "Infant, Newborn"] (14927) 14927
8. (Neonat\* OR newborn OR infant\* OR perinatal):ti,ab,kw (52650) 52650
9. #7 OR #8 (52650) 52650
10. #3 and #6 and #9 (55 hits) 64

Update search 13.3.21

1. [mh "Biomarkers"] OR [mh "Cytokines"] (33581) 38,098



2. (Biomarker\* OR 'biological indicator' OR 'biological marker' OR 'serum marker' OR 'Immunologic Marker' OR Cytokine\*):ti,ab,kw (23391) **59,632**
3. #1 OR #2 (48313) **76,305**
4. [mh "Hypoxia-Ischemia, Brain"] (149) **215**
5. (encephalopathy or 'Ischemia-Hypoxia' or 'Ischemia Hypoxia' or 'perinatal asphyxia' or 'fetal anoxia'):ti,ab,kw (2405) **4020**
6. #4 OR #5 (2405) **4020**
7. [mh "Infant, Newborn"] (14927) **16,143**
8. (Neonat\* OR newborn OR infant\* OR perinatal):ti,ab,kw (52650) **76,132**
9. #7 OR #8 (52650) **76,132**
10. #3 and #6 and #9 (55 hits) **112**
11. #3 and #6 and #9 **60**

## VII Chapter 6 Appendices

### Assessment of the data distribution for significance testing

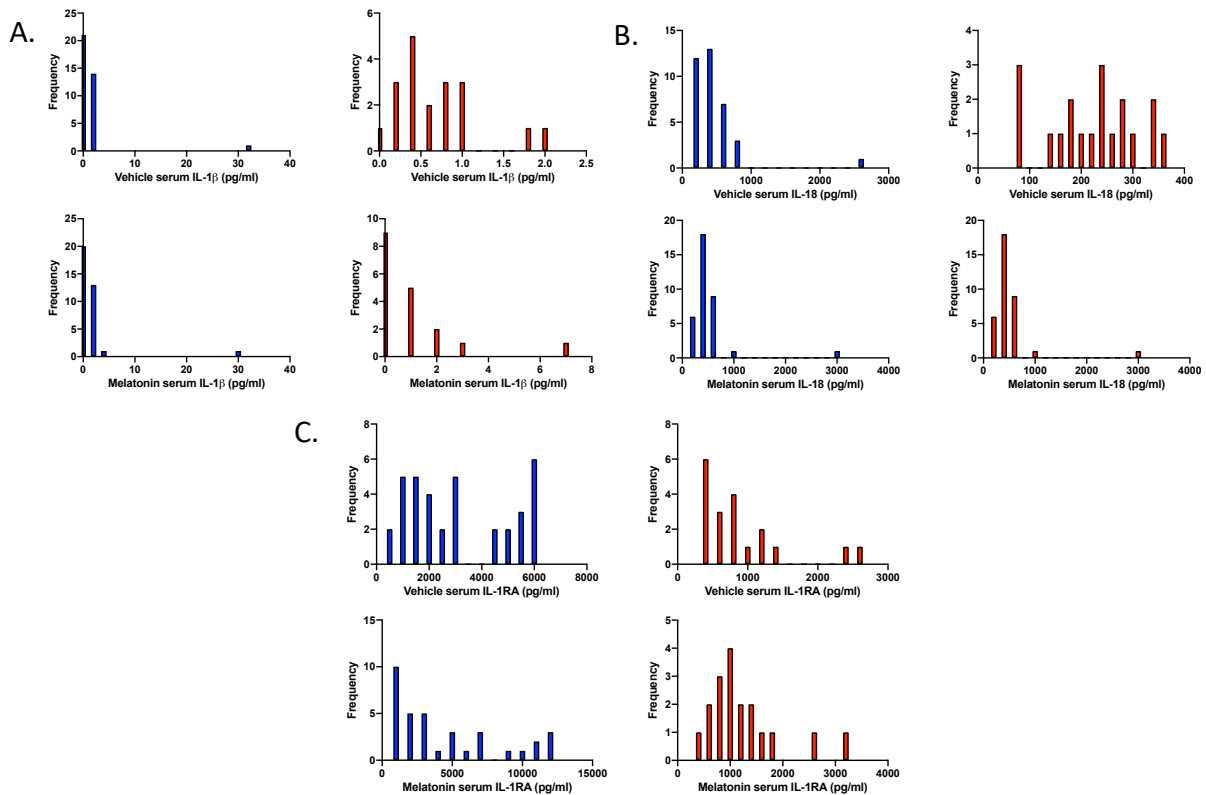


Figure 93 Histogram of the distribution of serum IL-1 receptor family cytokine concentration (pg/ml) in vehicle and melatonin treated samples from patients with NE and controls (A) IL1- $\beta$  (B) IL-18 (C) IL-1RA.

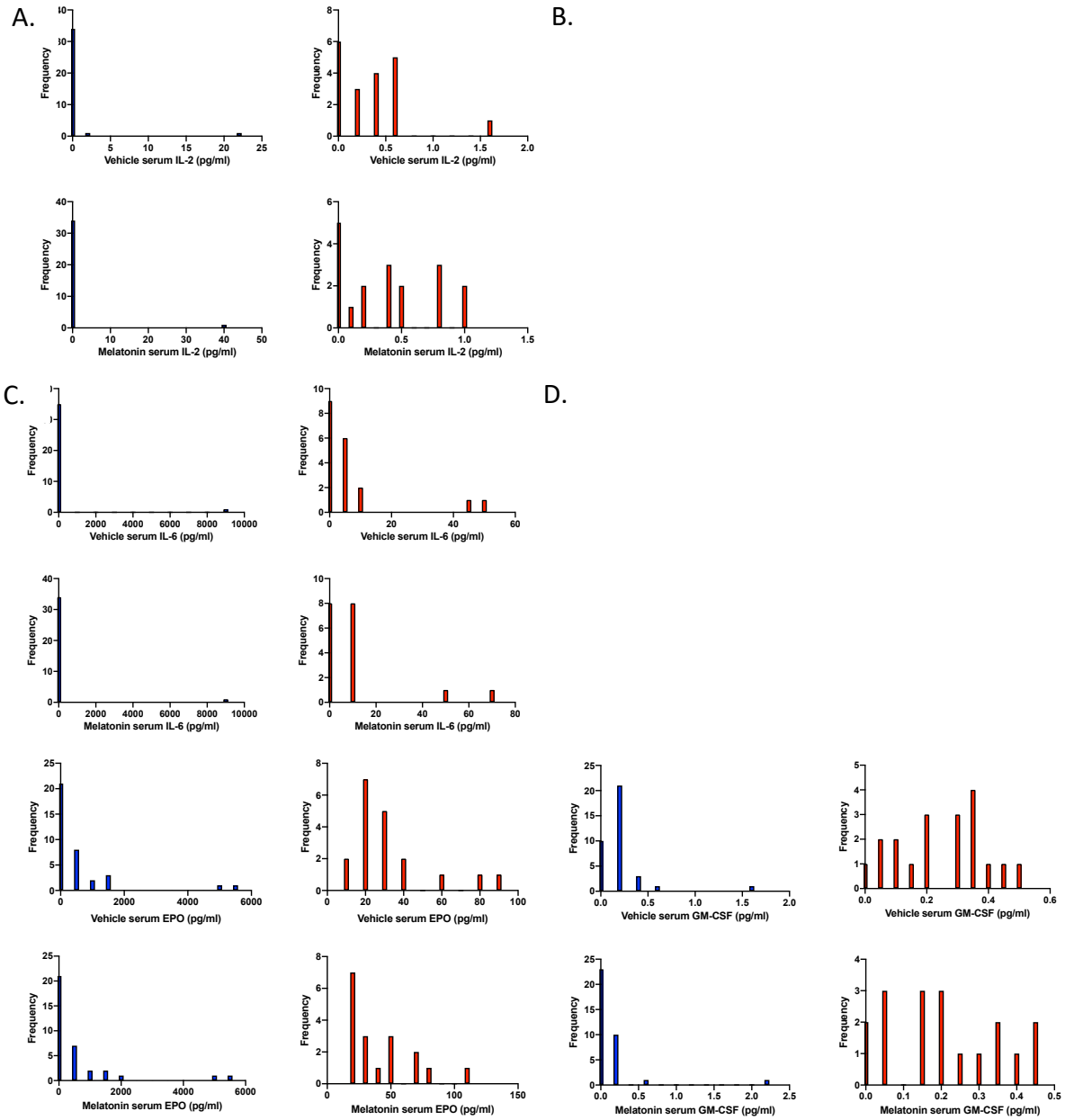


Figure 94 Histogram of the distribution of serum type 1 (haematopoietin) receptor family cytokine concentration (pg/ml) in vehicle and melatonin treated samples from patients with NE and controls (A) IL-2 (B) IL-6 (C) EPO (D) GM-CSF.

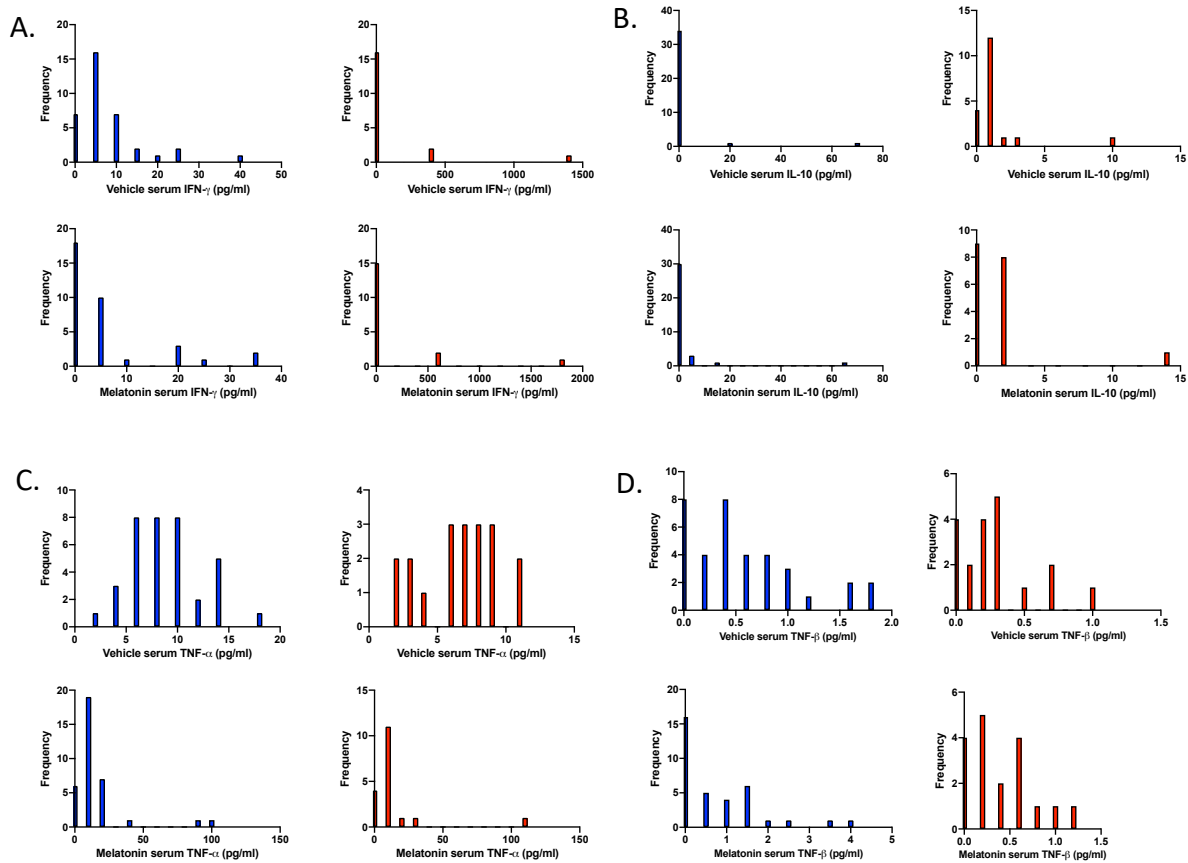


Figure 95 Histogram of the distribution of serum type 2 (interferon) receptor family & tumour necrosis factor family cytokine concentration (pg/ml) in vehicle and melatonin treated samples from patients with NE and controls (A) IFN- $\gamma$  (B) IL-10 (C) TNF- $\alpha$  (D) TNF- $\beta$ .

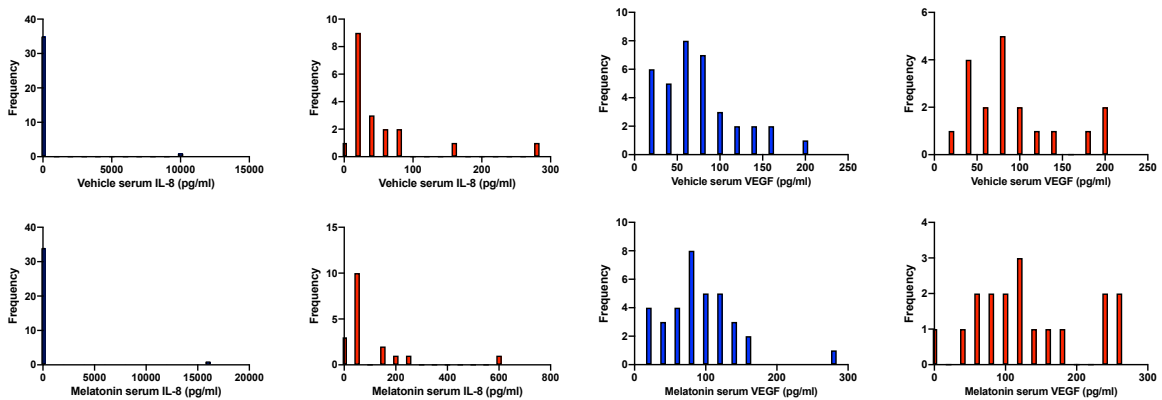


Figure 96 Histogram of the distribution of serum CXC chemokine family and vascular endothelial growth factor cytokine concentration (pg/ml) in vehicle and melatonin treated samples from patients with NE and controls (A) IL-8 (B) VEGF.

Cytokine	Vehicle NE	Vehicle Control	Mel NE	Mel Control
IL-1 $\beta$	<0.001	0.031	<0.001	<0.001
IL-18	<0.001	0.661	<0.001	0.289
IL-1RA	0.001	0.001	<0.001	0.008
IL-2	<0.001	0.002	<0.001	0.046
IL-6	<0.001	<0.001	<0.001	<0.001
EPO	<0.001	0.003	<0.001	0.005
GM-CSF	<0.001	0.712	<0.001	0.242
IFN- $\gamma$	<0.001	<0.001	<0.001	<0.001
IL-10	<0.001	<0.001	<0.001	<0.001
TNF- $\alpha$	0.826	0.442	<0.001	<0.001
TNF- $\beta$	0.004	0.014	<0.001	0.060
IL-8	<0.001	<0.001	<0.001	<0.001
VEGF	0.004	0.091	0.001	0.197

Table 35 Results of Shapiro-Wilk tests for normality of data distribution. The null hypothesis that the data are normality was rejected if the p value was <0.05.

## VIII Chapter 8 Appendices

A.

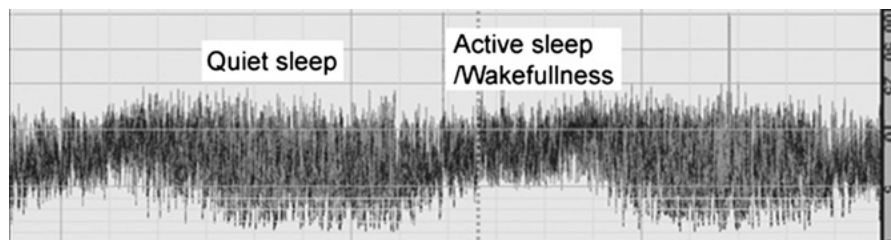


Figure 97 Illustration of different phases of electrographic sleep identified on aEEG recording per Takenouchi definition.

B.

Score	Continuity	Cycling	Amplitude of Lower Border	Bandwidth Span and Amplitude of Lower Border
0	Discontinuous	None	Severely depressed (<3 $\mu V$ )	Very depressed: low span ( $\leq 15 \mu V$ ) and low voltage (5 $\mu V$ )
1	Somewhat continuous	Waves first appear	Somewhat depressed (3–5 $\mu V$ )	Very immature: high span (>20 $\mu V$ ) or moderate span (15–20 $\mu V$ ) and low voltage (5 $\mu V$ )
2	Continuous	Not definite, somewhat cycling	Elevated (>5 $\mu V$ )	Immature: high span (>20 $\mu V$ ) and high voltage (>5 $\mu V$ )
3		Definite cycling, but interrupted		Maturing: moderate span (15–20 $\mu V$ ) and high voltage (>5 $\mu V$ )
4		Definite cycling, noninterrupted		Mature: low span (<15 $\mu V$ ) and high voltage (>5 $\mu V$ )
5		Regular and mature cycling		

Table 36 Summary of the cerebral maturation scoring system by Burdjalov et al.

C.

SCORE	FINDING
<b>BASAL GANGLIA &amp; THALAMUS</b>	
0	Normal or isolated focal cortical infarct
1	Abnormal signal in thalamus
2	Abnormal signal in thalamus and lentiform nucleus
3	Abnormal signal in thalamus, lentiform nucleus, and perirolandic cortex
4	More extensive involvement
<b>WATERSHED</b>	
0	Normal
1	Single focal infarction
2	Abnormal signal in anterior or posterior watershed white matter
3	Abnormal signal in anterior or posterior watershed cortex and white matter
4	Abnormal signal in both anterior and posterior watershed zones
5	More extensive cortical involvement

Table 37 Barkovich MRI brain scoring classification.

D.

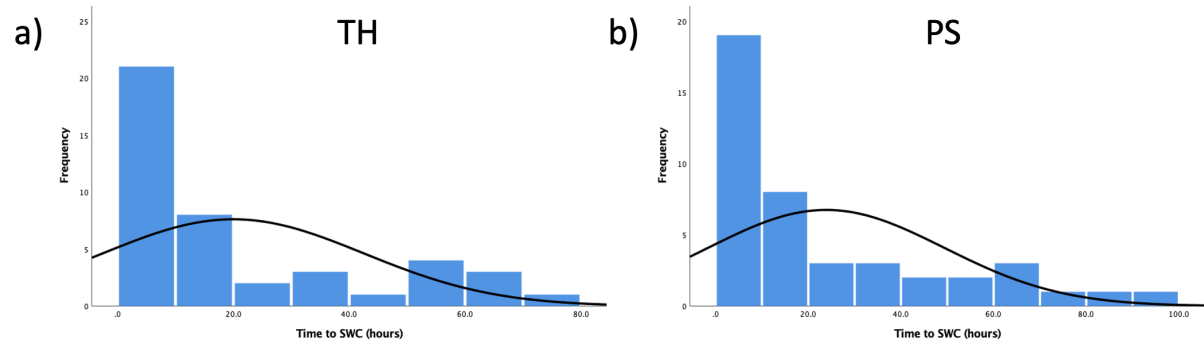


Figure 98 Histogram of the distribution of time to SWC in hours by (a)TH and (b)PS.

E.

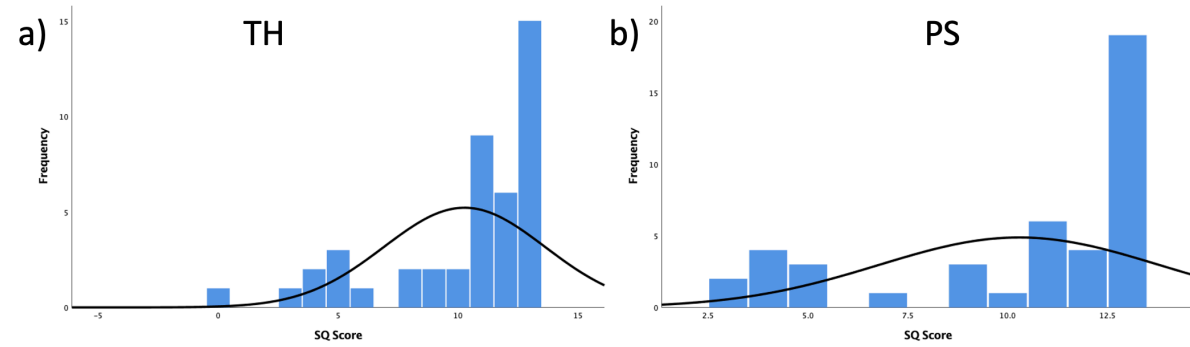


Figure 99 Histogram of the distribution of SQ score by (a)TH and (b)PS.

F.

Tests of Normality						
	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Time_to_SWC_Tim	.253	42	.000	.790	42	.000
Time_to_SWC_Philip	.206	42	.000	.813	42	.000
SQ_TotalScore_Tim	.256	42	.000	.787	42	.000
SQ_TotalScore_Philip	.255	42	.000	.758	42	.000

a. Lilliefors Significance Correction

Table 38 Kolmogorov-Smirnov test for normality of distribution of time to onset of SWC and SQ scores.

G.

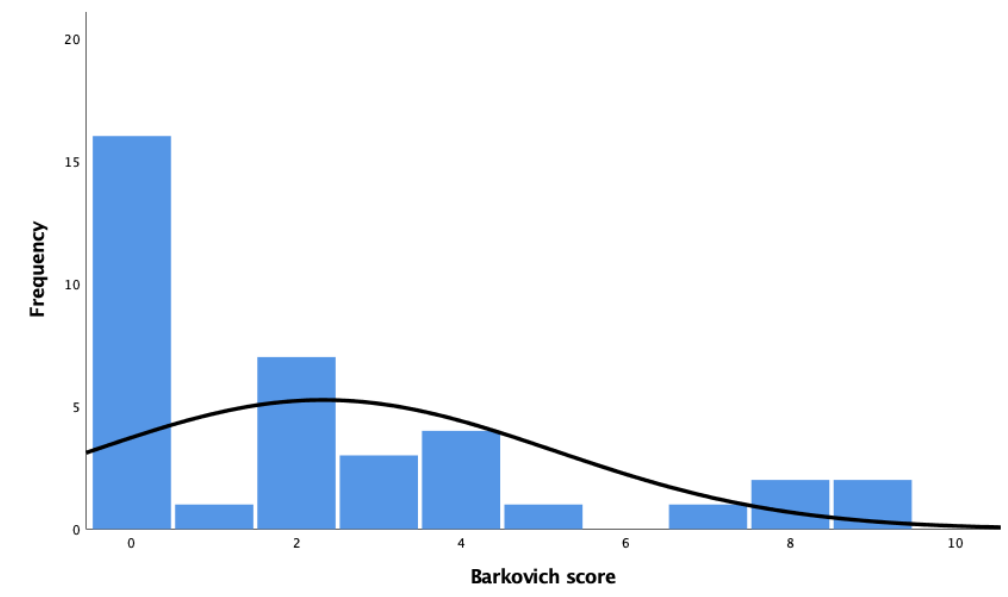


Figure 100 Histogram of the distribution of Barkovich score.

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